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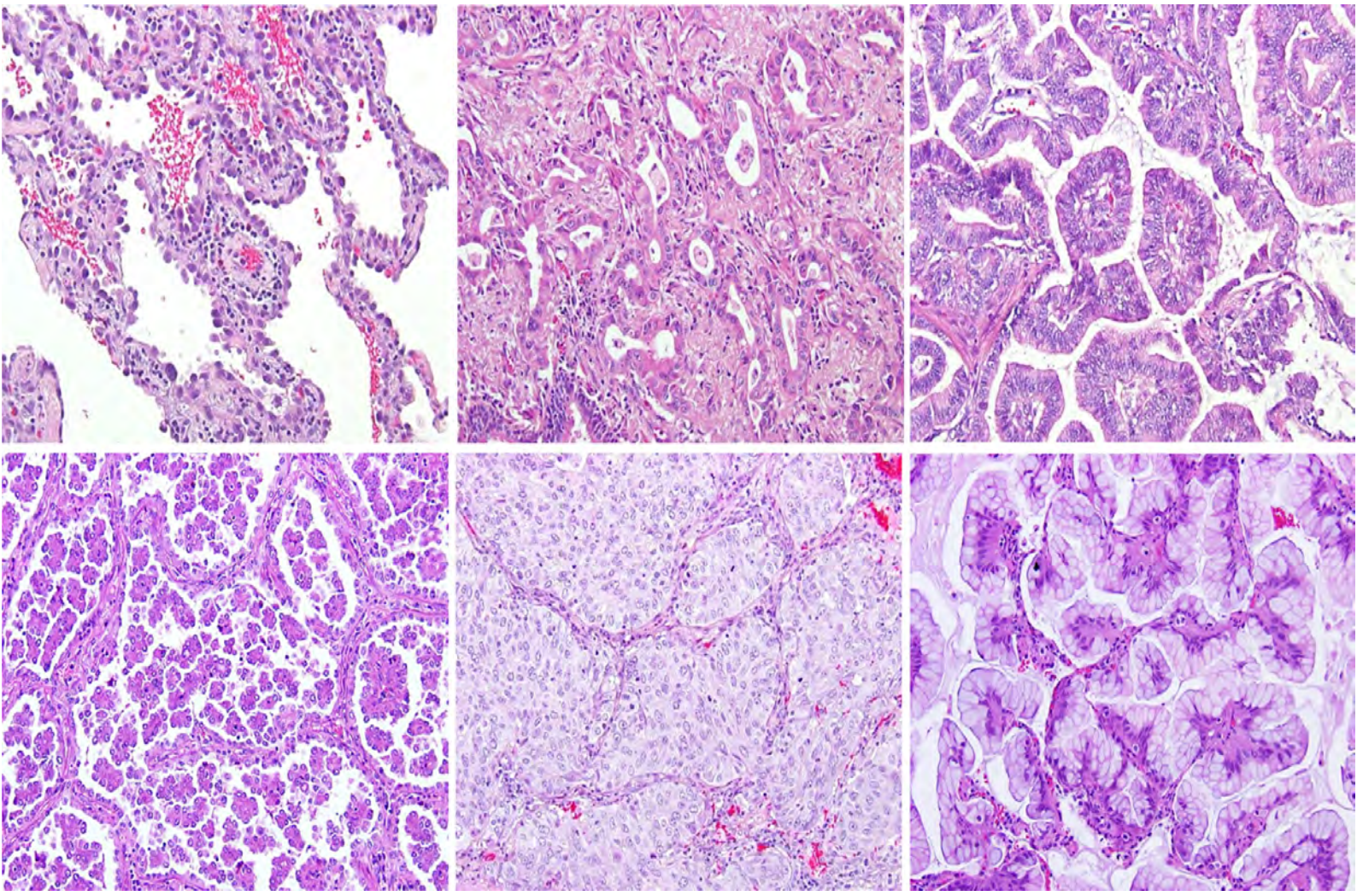
SCHOOL OF SCIENCE

# Biomedical Research Journal

APRIL 2014 | VOLUME 1 | ISSUE 1



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# Biomedical Research Journal

## General Information

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"Biomedical Research Journal (BRJ)" is a premier peer reviewed open access journal, published by NMIMS School of Science for promoting the advancement of ideas in the interdisciplinary realms of Medicine, Science and Technology. The goal is to share new discoveries and translational knowledge with scientists, academicians, clinicians and students in the field of Biomedical and Biological/ Chemical/ Biotechnology/ Stem Cell Biology/ Cancer Biology in the realm of basic and applied aspects in the different areas.

BRJ aims at creating a platform to help advance the domains and frontiers of inter- and multi-disciplinary research across the various areas of sciences and recent advances in cross pollination across biology, chemistry, and medicine. Integrative science is the present and future of science, and the various aspects of the journal proposes to highlight and emphasize contemporary technology towards understanding various aspects of the sciences.

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Contents

April 2014, Volume 1, Issue 1

BRJ
Biomedical Research Journal

Editorial

BRJ
Biomedical Research Journal

# Current Status of Cancer Burden: Global and Indian Scenario

Dhananjaya Saranath and Aparna Khanna

Today, cancer is a common household word, with each of us closely associated with at least one near and dear one, a family member or a friend, a neighbor or a colleague, diagnosed with cancer. In India, there is also a perception that cancer incidence is on the increase; and a hope that perhaps with the advances in technology, cancer is diagnosed more frequently, maybe a change in our attitude and approach, the myths associated with cancer are vanishing and we are more open to accepting cancer diagnosis and discussing cancer more openly. The first issue of our journal 'Biomedical Research Journal', we were fortunate to receive excellent articles from our colleagues, and coincidentally the focus of the articles was 'Cancer'. With the recent release in December 2013, of the latest data on cancer incidence, mortality and prevalence worldwide, by the International Agency for Research on Cancer (IARC), the specialized cancer agency of the World Health Organization, our editorial in this issue reflects a brief synopsis of some of the critical figures of cancers, globally and of relevance to our country.

IARC has published updated estimates for 28 types of cancer in 184 countries, giving a comprehensive overview of the global cancer burden in the Globocan 2012 report. We present a bird's eye view focusing on cancer statistics reported by IARC, globally and in India, to highlight the cancer burden as incidence, mortality and five-year prevalence. Thus, in 2012 IARC figures for global cancer burden estimates were 14.1 million new cases (with the 2008 estimates being 12.7 million new cases), 8.2 million cancer deaths, and five-year prevalence of 32.6 million cancers in individuals above the age of 15 years. Globocan 2012 estimates indicate a substantive increase to 19.3 million new cancer cases by 2025. It comes as no surprise that 57% (8 million) new cancer cases, 65% (5.3 million) cancer deaths, and 48% (15.6 million) five-year prevalence of cancer cases, occurred in the less developed regions of the world, a similar scenario in India.

On the Indian scene, 1.1 million new cancer cases were estimated, indicating India as a single country (of the 184 countries) contributing to 7.8% of the global cancer burden; mortality figures were 682830, contributing to 8.33% of global cancer deaths; and the five year prevalence was 1.8 million individuals with cancer corresponding to 5.52% of global prevalence.

Globally, the five most common cancers considered in both sexes were cancers of the lung (1,824,701; 13%), breast (1,676,633;

11.9%), colorectum (1,360,602; 9.7%), prostate (1,111,689; 7.9%), and cervix uteri (527,624; 3.7%), comprising 46.2% of the 28 cancers reported. Further, deaths due to these five cancers were 3,378,622. The estimated five most common cancers in men were cancers of lung (16.7%), prostate (15%), colorectum (10%), stomach (8.5%) and liver (7.5%) amounting to a total of 4,285,250 cancers, and death due to these cancers as 2,769,670. In women, the five most common cancers were cancers of breast (25.2%), colorectum (9.2%), lung (8.8%), cervix uteri (7.9%), and corpus uteri (4.8%) with a total of 3,721,266 cases. Death due to these cancers were 1,675,069. And the five year prevalence figures were 11.236 million.

On the Indian scene, the five most common cancers in both sexes were cancers of the breast (144,937; 14.3%), cervix uteri (122,844; 12.1%), lip-oral (77,003; 7.6), lung (70,275; 6.9%) and colorectum (64,332; 6.3%), comprising 47.2% of the 28 cancers reported. Further, death due to these five cancers are 302,124. The five most common cancers in men were cancers of lung (11.3%), lip-oral (11.3%), stomach (9.1%), colorectum (7.7%), and oro-pharynx (6.6%) resulting in 219,608 cancers; death due to these cancers were 180,670, with five-year prevalence as 235,840. The five most common cancers in women were cancers of breast (27%), cervix uteri (22.9%), colorectum (5.1%), ovary (5.0%) and lip-oral cavity (4.3%) with a total of 345,191 cancers; death due to these were amounted to 193,664, with the five-year prevalence as 833,106.

The take home message from the data on cancer incidence, mortality, and five-year prevalence should take into account the following:

Both globally and on the Indian scene, breast scene is dismal with more than 20% increase in breast cancer since 2008 with 1.7 million new cases diagnosed in women in 2012; and there were 6.3 million women alive with breast cancer in the previous five years. Breast cancer is also the most common cause of cancer deaths among women (521,817 deaths in 2012) and the most frequently diagnosed cancer among women in 140 of 184 countries worldwide. It now represents one in four of all cancers in women. For the first time, breast cancer is the leading cancer in Indian women and cause of cancer death, surpassing cervix uteri cancer, despite cervical cancer considered more common in rural India, and almost 80–85% of India is rural India.

Cervical cancer is the third most common cancer affecting women worldwide, the most common cancer in women in several less developed countries, and now the second most common cancer in India. Almost 70% of the global burden of cervical cancer is in areas with lower levels of development, and more than one fifth of all new cases are diagnosed in India. Low-tech and inexpensive screening tools, and the HPV DNA PCR testing available in several developed countries and low resource countries as well should be useful. Cervical cytology (Papanicolaou test or pap) has traditionally been the primary screening method for cervical cancer. However, the sensitivity of the test in several low resource

countries is rather poor (28–52%), and high risk HPV types are proven etiologic agents of cervical cancer. Cervical cancer screening would significantly reduce the burden of the disease and deaths associated with it. Recently, on Mar 12, 2014, US-FDA committee members recommended that detection of high-risk HPV, including genotyping for genotypes 16 and 18, be the first-line primary screening test for cervical cancer. Besides, the availability of prophylactic vaccines against the high risk HPV16/18 accounting for 70% cervical cancer across the world, may be the beginning of the end of cervical cancer. The need of the hour is to implement well planned and systematic cervical cancer screening programs and prophylactic HPV vaccination.

In males, the focus for reducing incidence, downgrading and treatment is most needed for lung cancer, a cancer due to the tobacco smoking habits highly prevalent worldwide. The cancer can be unequivocally combatted by change in lifestyle. Another tobacco associated cancer in India is oral cancer. However, all tobacco habitués do not show progression to the cancer, and hence an important risk often not taken into account is the genomic constitution of an individual developing the cancers. Attention needs to be focused on stomach and colorectal cancers as well.

The global focus to combat cancer needs to be on cancer awareness, early detection, diagnosis, and availability and affordability of treatment in all cancers.

In the current issue, we bring forth articles on the new international multidisciplinary histological classification of lung adenocarcinomas, with Dr. Adusumilli and colleagues from Memorial Sloan-Kettering Cancer Center, New York, USA. The classification with additional prognostic factors such as nuclear grade, cribiform pattern, immune and molecular markers enabled stratification of the risk of recurrence, adds value to clinical management of the patients. Ease of implementation on the Indian scene and validity for Indian patients is discussed by the authors.

Dr. Partha Basu and his team at Chittaranjan National Cancer Institute, Kolkata, India, elaborated on screening cervical cancers using aided visual inspection, recommended for limited resource countries, highlighting the advantages and hence the feasibility of use in several countries. Besides, the authors advocate "screen and treat" approach of practical value, although the article does point out the disadvantages of low specificity and sub-optimal positive predictive value of the test. Perhaps a thought from the editors, why short change the women in low resource setting and instead provide them access to HPV detection and genotyping, a highly specific and sensitive test for primary screening of cervical cancer, via availability, affordability and subsidy through health systems in corporates, non-government organizations and government-based subsidy. Is it fair to lose women productivity and life because of cervical cancer in the 21$^{st}$ century?

We have Dr. Pritha Ray and her colleagues from Advanced Centre for Treatment, Research and Education in Cancer, Navi

Mumbai, India, and Tata Memorial Hospital, Mumbai, India, with an article on ovarian cancer, a heterogenous disease, and fourth most common cancer in India. The authors review and detail the cancer stages, and indicate the possible use of biomarkers including CA-125, useful in differential diagnosis and follow up, but not an effective marker for early-stage ovarian cancer due to its unacceptable low sensitivity and specificity. Ovarian cancer usually has a relatively poor prognosis. It is disproportionately deadly because it lacks early detection or screening tests, implying that most cases are not diagnosed until they have reached advanced stages. Possible molecular markers include micro-RNAs, methylation markers, and ultrasonography and computed sonography may facilitate early diagnosis. Current studies on proteomics mark the beginning of a paradigm shift towards individually tailored therapy. The authors expand on current treatment for the cancer. The need for better targeted therapy and newer imaging modalities are seen as possible solutions for management of ovarian cancer patients.

The article by Dr. Narendra Joshi, ACTREC, Navi Mumbai, India, focused on 'single nucleotide polymorphisms (SNPs) in human health and disease', is indeed very apt in the current context. An amazing aspect of the human genome is the minimal variation in the DNA sequence in the genome of different individuals. Of the 3.2 billion bases, roughly 99.9% are identical between two individuals. It is the variation in the remaining tiny fraction of the genome, 0.1%, that makes a person unique. This small amount of variation determines critical attributes of the individual in developing cancer and response to treatment. Besides the well-known lifestyle risk factors such as tobacco, alcohol, infectious agents - HPV, HBV, as mentioned earlier a very critical risk factor is the genomic constitution of an individual. We have all amply observed individuals who drink alcohol and smoke cigarettes live to the ripe age of 90 years without getting liver or lung cancer; whereas, some individuals who smoke and drink the same amount get liver/lung cancer at age 60 years or earlier; and still interesting is the development of cancer at age 55 years in individuals without either of the habits. Additionally, response to chemotherapy for almost every cancer is not identical, with one individual responding to chemotherapy, with shrinking of the cancer; whereas, another comparable individual's identical cancer shows no change after the same treatment. Thus, progression to cancer with/without risk factor, and response to treatment, is dependent on the genomic constitution of an individual. Dr. Joshi takes us through understanding the genomic variants as SNPs, and its implications and role in cancer development. The SNPs may play a role in the different responses to treatment observed in cancer patients. The author emphasizes the importance of single nucleotide polymorphisms in health and human diseases, including cancer. Thus, SNPs may be promising potential tools to better cancer diagnosis and treatment planning. SNP data may reveal markers for predisposition to cancer.

The genome of each individual contains its own pattern of SNPs. Thus, each individual has his or her own SNP profile. When scientists look at all the patterns from a large group of individuals, they can organize them into groups. Armed with data from the SNP Map, cancer research groups are focusing on correlations between SNPs and precancerous conditions, SNPs and drug resistance in chemotherapy, SNPs and cancer susceptibility, and SNPs and drug responses.

Genetic variation in the human genome is an emerging resource for studying cancer, a complex set of diseases characterized by both environmental and genetic contributions.

The final original research article from Dr. Anjali Karande, Indian Institute of Science, Bangalore, India, focusses on GnRH receptors in breast cancer. The authors have investigated the GnRH receptors on breast cancer cell line and demonstrated that GNRH analogue inhibits proliferation of the cancer cell line, via inhibition of processes that trigger cAMP formation. The translation of cancer research from bench to bedside may ultimately unravel the mysteries of human cancers and lead us to better patient management.

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BRJ
Biomedical Research Journal

# The New International Multidisciplinary Histological Classification of Lung Adenocarcinoma and Clinical Implications for Indian Physicians

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In 2011, a new histologic classification of lung adenocarcinomas (IASLC/ATS/ERS) was proposed based on the recommendations of an international and multidisciplinary panel that included thoracic medical oncologists, pulmonologists, radiologists, molecular biologists, thoracic surgeons and pathologists. This classification proposed a comprehensive histologic subtyping (lepidic, acinar, papillary, micropapillary and solid pattern) and a semi-quantitative assessment of histologic patterns (in 5% increments) in an effort to choose a single, predominant pattern. The prognostic value of this classification has been validated in large, independent cohorts from multiple countries. Patients with adenocarcinomas *in situ* and minimally invasive adenocarcinomas experienced no recurrence. Patients with micropapillary or solid predominant tumors would be classified as high risk for recurrence or cancer-related death. Patients with acinar and papillary predominant tumors might be classified as an intermediate-risk group, but further investigation is needed for papillary subtype. This classification, coupled with additional prognostic factors (nuclear grade, cribriform pattern, high Ki-67 labeling index, TTF-1 negativity, immune markers and SUVmax on FDG-PET), which we have published on extensively, could further stratify patients into prognostic subgroups that may help with clinical management. This new classification for the most common type of lung cancer is important for oncologists practicing in India, as its implementation would require only hematoxylin and eosin (H&E) histology slides, the most common type of stain used at hospitals. It can be implemented with basic pathologist training and no additional costs. Furthermore, implementation and analyses would identify if this classification is valid for Indian patients or a specific modification is required.

## INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide (Jemal *et al*., 2011; Siegel *et al*., 2012). Over the past decade, the rate of adenocarcinoma (the most frequent subtype of lung cancer) has increased in a majority of the countries (Devesa *et al*., 2005; Youlden *et al*., 2008). Currently, the single most important factor that determines prognosis for patients with lung adenocarcinomas is tumor-nodal-metastasis (TNM) stage (Edge *et al*., 2009). Lung adenocarcinoma is a heterogeneous tumor with great variation in pathological profile. Histologic classifications of lung cancers have been published by the World Health Organization (WHO) in 1967, 1981, 1999 and 2004, and the most recent revision has introduced relevant clinical and molecular genetic information (Travis *et al*., 2004). Despite the updating, there is limited clinical

**Key words:** Lung, adenocarcinoma, histological classification, prognosis.
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utility in the 2004 WHO classification of lung adenocarcinomas, since more than 90% of adenocarcinomas are classified as a mixed subtype even though they have a wide variety of clinical outcomes (Motoi *et al*., 2008; Travis *et al*., 2011; Yoshizawa *et al*., 2011). Increasing evidence suggests that histologic pattern scan identifies significant prognostic subsets of patients with lung adenocarcinomas (Barletta *et al*., 2010; Borczuk *et al*., 2009; Motoi *et al*., 2008; Nakazato *et al*., 2010; Sica *et al*., 2010; Yim *et al*., 2007). Multiple studies have shown that patients with pure lepidic (noninvasive) adenocarcinomas had 100%, 5-year disease-free survival (Koike *et al*., 2009; Noguchi *et al*., 1995; Sakurai *et al*., 2004; Vazquez *et al*., 2009). Other studies showed that patients with lepidic predominant minimally invasive (≤ 5 mm invasion) adenocarcinomas had near 100% survival (Borczuk *et al*., 2009; Maeshima *et al*., 2010; Yim *et al*., 2007). Lepidic predominant invasive tumors also correlate with a favorable prognosis in patients with resected lung adenocarcinomas (Lee *et al*., 2009; Lin *et al*., 2006; Yokose *et al*., 2000). In contrast, the micropapillary pattern bodes a poor prognosis in patients with lung adenocarcinomas (Miyoshi *et al*., 2003; Nagano *et al*., 2010). In the WHO classification, the diagnostic criteria are based primarily on H&E examination with recognition of importance of integration of immunohistochemical, histochemical, and molecular studies. To address the advances in the prognostic pathological findings identified over the last decade, a new histologic classification is needed to provide histological subtypes with uniform terminology and diagnostic criteria.

In addition to the pathologic findings that define prognosis, advances in radiologic-pathologic correlations, molecular biology, and thoracic medical oncology for lung adenocarcinomas over the past decade have been reported. On chest computed tomography (CT) of lung adenocarcinomas, the correlations between lepidic growth and ground-glass opacity and between invasive components and solid components, have been identified and predict histologic subtypes for patient prognosis. CT has also been used for improving preoperative clinical decision-making of surgical procedures (i.e., lobectomy versus limited resection) (Nakata *et al*., 2003; Okada *et al*., 2006; Suzuki *et al*., 2002; Takashima *et al*., 2002).

Recent advances in molecular biology in conjunction with medical oncology have demonstrated that activating mutations in the tyrosine kinase domain of *epidermal growth factor receptors* (*EGFR*) predicts responsiveness to *EGFR* tyrosine kinase inhibitors (TKI) in patients with non-small cell lung cancer (NSCLC) (Lynch *et al*., 2004; Paez *et al*., 2004; Pao *et al*., 2004). The mutations are most frequently observed in females, in non-smokers and in Asian patients with adenocarcinomas (Lynch *et al.,* 2004; Paez *et al*., 2004; Pao *et al*., 2004; Shigematsu *et al*., 2005; Tam *et al*., 2006). *EGFR* mutations have also been associated with lepidic pattern adenocarcinomas, formerly known as bronchioloalveolar carcinoma (BAC) patterns (Blons *et al*., 2006; Hsieh *et*

*al*., 2005; Marchetti *et al*., 2005; Tam *et al*., 2006). The association led to the hypothesis that tumors with lepidic pattern adenocarcinomas may be correlated with the *EGFR* mutations and may predict responses to TKI (Kim *et al*., 2004; Miller *et al*., 2004; Zakowski *et al*., 2009). In addition, incidence of the specific secondary EGFR mutation (T790M) is the main molecular mechanism responsible for acquired resistance to EGFR-TKIs (Suda *et al*., 2010; Yun *et al*., 2008). *Kirsten rat sarcoma viral oncogene homolog* (*KRAS)* is one of the downstream molecules in the *EGFR* signaling pathway (Mitsudomi and Yatabe, 2007; Riely *et al*., 2009). In contrast to *EGFR* mutations, *KRAS* mutations predict resistance to TKI treatment in patients with NSCLC (Eberhard *et al*., 2005; Pao *et al*., 2005) and are correlated with a history of cigarette smoking and poor patient prognosis (Ahrendt *et al*., 2001; Marks *et al*., 2008; Mascaux *et al*., 2005; Tam *et al*., 2006). In addition, the *KRAS* mutation shows a correlation with invasive mucinous adenocarcinoma, formerly known as mucinous BAC (Casali *et al*., 2010; Finberg *et al*., 2007; Hata *et al*., 2010; Kakegawa *et al*., 2011; Marchetti *et al*., 1996). A recently discovered anaplastic lymphoma kinase (ALK) rearrangement predicts sensitivity to a targeted agent (Crizotinib) (Kwak *et al*., 2010; Shaw *et al*., 2009). *ALK* rearrangements exclusively occur in adenocarcinomas and are correlated with specific histological findings such as signet-ring cell features, extracellular mucin, and cribriform patterns (Inamura *et al*., 2009; Jokoji *et al*., 2010; Rodig *et al*., 2009).

**International Association for the Study of Lung Cancer (IASLC)/American Thoracic Society (ATS)/European Respiratory Society (ERS) histologic classification of lung adenocarcinoma: Historical perspective**

To provide an international and multidisciplinary approach to the development of a new histologic classification system for identifying prognostic subtype, the IASLC/ATS/ERS selected thoracic medical oncologists, pulmonologists, radiologists, molecular biologists, thoracic surgeons, and pathologists as panel members, based on their special interest and expertise in lung adenocarcinoma (Travis *et al*., 2011). The panel performed a systematic review of the literature on lung adenocarcinoma and generated a series of key questions by specialty. The search strategy initially yielded 11,368 relevant articles. Of these, 312 met the specified eligibility criteria for a full-text review. After review, and in conjunction with each specialty group, a writing committee developed the recommendations for histologic classification. Following a multidisciplinary discussion between 2008 and 2009, this classification system was subsequently modified, and separate projects were initiated by the panel members in an effort to validate the proposed system (Sica *et al*., 2010; Thunnissen *et al*., 2012; Yoshizawa *et al*., 2011). On the basis of this multidisciplinary approach, the panel recommended 10 significant changes to the diagnostic classification of lung adenocarcinomas in order to improve precision in predicting

clinical outcome and therapeutic benefits. These recommendations are detailed in the 2011 joint publication by the IASLC, ATS, and ERS, proposing the new classification system (Travis *et al*., 2011).

**The 2011 IASLC/ATS/ERS lung adenocarcinoma histologic classification**

The IASLC/ATS/ERS lung adenocarcinoma histologic classification system was proposed in the *Journal of Thoracic Oncology* in 2011 (Travis *et al*., 2011). According to this new classification, a tumor should be reviewed by using comprehensive histological subtyping, recording the percentage of each histological component (lepidic, acinar, papillary, micropapillary, or solid) in 5% increments and choosing a single predominant pattern. In addition, total tumor size and invasive tumor size are measured. Tumor invasion was defined as: (1) histologic pattern other than lepidic (acinar, papillary, micropapillary or solid); (2) active myofibroblastic stroma correlated with invasive tumor cells; and (3) presence of lymphatic, vascular, or pleural invasion. Invasive tumor size was measured in two distinct ways comprising (1) cases where the tumor was small and the invasive area could be measured on a single slide, the invasive size was measured at either ×20 or ×40 magnification on the microscope, using a ruler; and (2) cases where the tumor was large and the invasive area could not be measured on a single slide, the invasive size was calculated by multiplying the total tumor size by the percentage of the invasive component (Yoshizawa *et al*., 2011). Adenocarcinoma *in situ* (AIS) is defined as a ≤ 3 cm tumor with a pure lepidic pattern, but without lymphatic, vascular, or pleural invasion or tumor necrosis. Minimally invasive adenocarcinoma (MIA) is defined as a ≤ 3 cm tumor with a lepidic predominant pattern and ≤ 5 mm stromal invasion, but with no lymphatic, vascular, or pleural invasion or tumor necrosis. It is important to understand that AIS and MIA should only be considered for diagnosis when a tumor is completely resected and the entire tumor area is histologically investigated (Travis *et al*., 2011). In addition, the CT findings and gross appearance of a tumor should be considered to ensure that a solid component is sampled from the tumor that appeared to be solid on the CT scan (Travis *et al*., 2011). Lepidic growth was classified into two patterns, nonmucinous and mucinous, according to the absence or presence of an intracellular mucinous feature. AIS and MIA were further subgrouped as nonmucinous, mucinous, or mixed mucinous/nonmucinous. Invasive adenocarcinomas (> 5 mm invasion size) were further divided into lepidic predominant (Figure 1A), acinar predominant (Figure 1B), papillary predominant (Figure 1C), micropapillarypredominant (Figure 1D), solid predominant (Figure 1E), invasive mucinous adenocarcinoma (Figure 1F), colloid predominant adenocarcinoma, enteric adenocarcinoma and fetal adenocarcinoma (low and high grade).

Application of the IASLC/ATS/ERS adenocarcinoma classification to biopsy or cytologic specimens is challenging as these

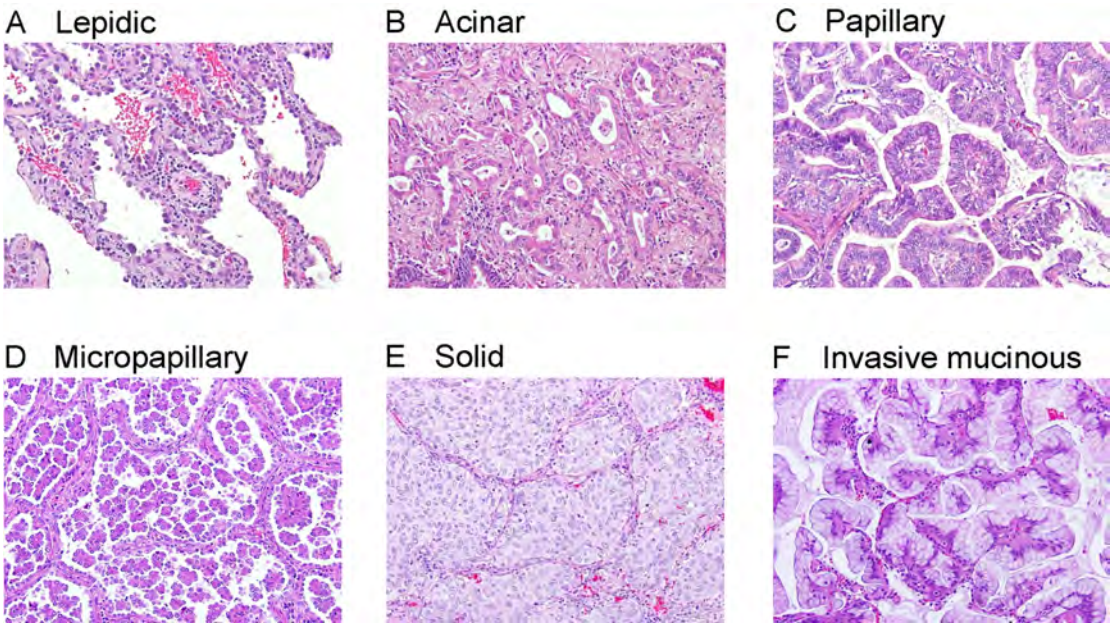


**Figure 1. Histologic pattern (hematoxylin and eosin stain; original magnification: ×100 magnification).**
(A) Non-mucinous lepidic pattern. (B) Acinar pattern. (C) Papillary pattern. (D) Micropapillary pattern. (E) Solid pattern. (F) Invasive mucinous pattern.

may not be representative of the total tumor due to histologic heterogeneity; there may also be a discrepancy between the initial and the final histologic diagnoses in a resection specimen (Travis *et al*., 2011). A recent study suggested that it may be unreliable and difficult to identify high grade histologic (solid and micropapillary) pattern on cytology specimens (Rodriguez *et al*., 2013). However, nuclear grade on cytologic specimens may provide support for the classification of tumors into different prognostic groups preoperatively (Sigel *et al*., 2012). This will be used in conjunction with the IASLC/ATS/ERS adenocarcinoma classification.

The IASLC/ATS/ERS histologic subtyping is primarily validated for early-stage lung adenocarcinomas. Testing for *EGFR* mutation in patients with advanced lung adenocarcinomas is also recommended by the IASLC/ATS/ERS classification because response and outcome to *EGFR*- TKIs can be predicted by presence of an *EGFR* mutation (Travis *et al*., 2011). According to the guidelines proposed by the College of American Pathologists, in order to do accurate testing for *EGFR* mutation, laboratories should use testing methods that allow the detection of mutation in specimens with ≥ 50% cancer cell content. Despite this, laboratories are strongly encouraged to use more sensitive tests thatare able to detect mutations in specimens with as little as 10% cancer cells (Lindeman *et al*., 2013). Regarding molecular findings, there may be important differences between the primary tumor and metastases of lung adenocarcinomas. However, further investigations are needed as the mutation status of metastases is unpredictable between

**Table 1.** Published studies validating the IASLC/ATS/ERS lung adenocarcinoma classification (study sample > 300 patients)

| Histologic subtype | United States (N = 514) | | Germany (N = 500) | | Japan (N = 440) | | Japan (N = 904) | |
|---|---|---|---|---|---|---|---|---|
| | % of patients | DFS % (5-yr) | % of patients | DFS (mean) | % of patients | DFS % (5-yr) | % of patients | OS % (5-yr) |
| AIS | 0.2 | 100 | 0.0 | NA | 4.5 | 100 | 7.6 | 98 |
| MIA | 1.6 | 100 | 0.0 | NA | 7.5 | 100 | 3.7 | |
| Lepidic | 5.6 | 90 | 8.2 | 72.6 mo. | 8.2 | 94 | 15.0 | 93 |
| Acinar | 45.1 | 84 | 41.4 | 61.7 mo. | 13.9 | 70 | 10.8 | 67 |
| Papillary | 27.8 | 83 | 4.6 | 37.7 mo. | 40.7 | 67 | 37.4 | 74 |
| Micropapillary | 2.3 | 67 | 6.6 | 33.8 mo. | 4.3 | 0 | 6.7 | 62 |
| Solid | 13.0 | 70 | 36.6 | 51.2 mo. | 17.7 | 43 | 13.7 | 58 |
| Inv. mucinous | 2.5 | 76 | 2.4 | 88.1 mo. | 2.3 | 89 | 5.0 | 76 |
| Colloid | 1.8 | 71 | 0.0 | NA | 0.7 | NA | 0.0 | NA |
| Enteric | 0.0 | NA | 0.2 | NA | 0.0 | NA | 0.0 | NA |
| Fetal | 0.0 | NA | 0.0 | NA | 0.2 | NA | 0.0 | NA |

DFS, disease-free survival; OS, overall survival; NA, not applicable

primary tumors and metastases (Travis *et al*., 2011).

Moreover, specific treatments (i.e., gefitinib, bevacizumab and pemetrexed) should be guided by histological types (adenocarcinoma versus squamous cell carcinoma) on the basis of dramatic advances in thoracic medical oncology over the past few years. Hence, IASLC/ATS/ERS classification recommends using immunohistochemistry to classify lung cancers into adenocarcinomas or squamous cell carcinomas; this being especially true if the tumor cannot be classified by light microscopy alone (Travis *et al*., 2011). As for the diagnostic markers, thyroid transcription factor-1 (TTF-1) and Napsin A have been validated as adenocarcinoma markers, and p40 and p63 as squamous cell carcinoma markers (Bishop *et al*., 2012; Rekhtman *et al*., 2011; Turner *et al*., 2012).

## Validation studies of the 2011 IASLC/ATS/ERS lung adenocarcinoma histologic classification

We summarized the published studies that validated the 2011 IASLC/ATS/ERS lung adenocarcinoma histologic classification using a large cohort (more than 300 patients as a study sample) in Table 1 (Tsuta *et al*., 2013; Warth at al., 2012; Yoshizawa *et al*., 2011; Yoshizawa *et al*., 2013). There was one study from the United States (n = 514) (Yoshizawa *et al*., 2011), one from Germany (n = 500) (Warth *et al*., 2012), and two from Japan (n = 440 and 904) (Tsuta *et al*., 2013; Yoshizawa *et al*., 2013). The study from the United States validated the new classification by using a homogeneous cohort composed of only stage I

patients (Yoshizawa *et al*., 2011), while the other studies comprised of patients with both early and advanced stage diseases (Jokoji *et al*., 2010; Rodriguez *et al*., 2013; Thunnissen *et al*., 2012).

**Other prognostic factors not included in the 2011 IASLC/ATS/ERS lung adenocarcinoma histologic classification**

According to the aforementioned large cohort validation studies, the 2011 IASLC/ATS/ERS lung adenocarcinoma histologic classification has great prognostic value (Tsuta *et al*., 2013; Yoshizawa *et al*., 2011; Yoshizawa *et al*., 2013). Despite this, there was a limitation of this classification that was identified. The limitation was that the majority of tumors (50–70%) classified as having intermediate grade histology (acinar or papillary predominant subtype) may actually include a heterogeneously prognostic subgroup. We propose that another prognostic factor, preferably one based on morphological analysis, is needed for this majority group. We have recently published studies that discuss the use of several prognostic factors that are based on morphological analysis, such as histologic findings (nuclear feature, cribriform subtype, and presence of micropapillary pattern), immunohistochemical analysis (Ki-67 labeling index and TTF-1), immune markers (tumor-infiltrating lymphocyte and cytokine receptor expression) and radiologic biomarkers (maximum standard uptake value [SUVmax] on 18F-fluorodeoxyglucose [FDG] uptake on positron emission tomography [PET]), when investigating a large cohort comprising stage I lung adenocarcinoma patients (Kadota *et al*., 2012a; Kadota *et al*., 2012b; Kadota *et al*., 2013a; Kadota *et al*., 2013b; Nitadori *et al*., 2013; Suzuki *et al*., 2013).

Using a cohort of stage I lung adenocarcinoma patients, we evaluated all the nuclear features including nuclear diameter, nuclear atypia, nuclear/cytoplasmic ratio, chromatin pattern, prominence of nucleoli, intranuclear inclusions, mitotic count, and atypical mitoses, and identified nuclear diameter, nuclear atypia, mitotic count, and atypical mitoses as predictors of risk of recurrence (Kadota *et al*., 2012b). Among these features, we discovered that mitotic count was an independent risk factor of recurrence. Using this information, we established a combined architectural (based on the 2011 IASLC/ATS/ERS classification) and mitotic count grading system. This new system was able to better stratify patients for risk of recurrence when compared with stratification done using the 2011 IASLC/ATS/ERS classification *per se*.

We also reported the prognostic significance of the cribriform pattern as a predominant subtype. In addition to the 2011 IASLC/ATS/ERS classification, we proposed using this as a distinct histologic subtype with poor prognosis (Kadota *et al*., 2013b). The recurrence-free probability for patients with cribriform predominant tumors was significantly lower than it was for patients with acinar or papillary predominant tumors; however, it was comparable to the probability for patients with micropapillary or solid

predominant tumors. These findings give credence to the hypothesis that cribriform pattern was an independent prognostic factor.

We further investigated the prognostic significance of the histologic pattern in small (≤ 2 cm) stage I lung adenocarcinoma patients who underwent different surgical procedures (limited resection vs. lobectomy). We identified the presence (≥ 5%) of micropapillary patterns as the risk factor of recurrence in patients treated with limited resection but not for those treated with lobectomy (Nitadori *et al*., 2013). Interestingly, in the limited resection group, tumors that presented a micropapillary pattern correlated with locoregional recurrence.

In addition to the mitotic count, Ki-67 also represents proliferation of tumor cells. Based on immunohistochemical analysis using tissue microarrays on stage I lung adenocarcinomas, we reported a high Ki-67 labeling index (threshold, 10%) which was indicative of it being a predictor of recurrence (Kadota *et al*., 2012b). Whereas, TTF-1 is known as a positive diagnostic marker for differentiating between lung adenocarcinomas and squamous cell carcinomas, TTF-1 negativity is an independent risk factor of recurrence in stage I lung adenocarcinomas (Kadota *et al*., 2013a). More importantly, tumoral TTF-1 expression status further stratified patients with intermediate grade tumors (acinar and papillary predominant subtype) based on their risk of recurrence.

Recent evidence suggests that the immune microenvironment also has prognostic significance in solid cancers (Galon *et al*., 2006; Mahmoud *et al*., 2011). We investigated the prognostic significance of tumor-infiltrating immune cells in tumor and tumor-related stroma, tumoral cytokine and cytokine receptor expression via immunohistochemical analysis using tissue microarrays in 2 large, independent cohorts (training and validation; n = 478 each) of patients with stage I lung adenocarcinomas. We identified high forkhead box P3 (FoxP3)/CD3 lymphocyte infiltration ratio in tumor-related stroma, tumoral interleukin-7 receptor (IL-7R) overexpression, and a loss of IL-12Rβ2 expression as poor independent prognostic indicators of recurrence (Suzuki *et al*., 2013). All of these immune markers were able to further stratify the risk of recurrence in each histological grade based on the 2011 IASLC/ATS/ERS classification.

SUVmax on FDG-PET has been recognized as a prognostic factor in lung cancer. Accordingly, we investigated the prognostic value of SUVmax on FDG-PET in patients with stage I lung adenocarcinoma (Kadota *et al*., 2012a). High SUVmax (≥ 3.0) was associated with a poor prognosis of recurrence and it further stratified the risk of recurrence in patients with intermediate grade histology (acinar or papillary predominant tumors). We observed that a high SUVmax correlated with high grade histology based on the 2011 IASLC/ATS/ERS classification.

**Future potential of the 2011 IASLC/ATS/ERS classification**

As stated earlier, the use of the 2011 IASLC/ATS/ERS classification as a powerful

prognostic evaluator has been validated many times over by large, independent data sets from multiple countries (Jokoji *et al*., 2010; Motoi *et al*., 2008; Rodriguez *et al*., 2013; Thunnissen *et al*., 2012). Recently, our group has proposed the use of other prognostic factors, in addition to those already used by the 2011 IASLC/ATS/ERS classification, based on a study of a large, homogeneous cohort comprising patients with stage I lung adenocarcinomas (Kadota *et al*., 2012a; Kadota *et al*., 2012b; Kadota *et al*., 2013a; Kadota *et al*., 2013b; Nitadori *et al*., 2013; Suzuki *et al*., 2013). CT scans of lung adenocarcinomas have suggested a correlation between ground-glass opacity (an air density-containing area on a CT scan) and lepidic growth patterns (Nakata *et al*., 2003; Suzuki *et al*., 2002; Takashima *et al*., 2002). Results from the recent randomized trials assessing low-dose CT screening for lung cancer (Aberle *et al*., 2011a; Aberle *et al*., 2011b; van lersel *et al*., 2007) suggest that an increasing number of patients will be diagnosed with adenocarcinomas with lepidic growth at an early stage. This may ultimately contribute to a reduced disease-related mortality rate for those types of patients in the future. Hence, it is important to recognize the clinical characterization of early-stage lung adenocarcinoma with lepidic predominant pattern. Since AIS and MIA are curable if completely resected, it is of interest to surgeons considering limited resection over standard lobectomy as a treatment option.

While several previous clinical trials applied adjuvant chemotherapy to stage I NSCLC patients, the treatment yielded no clinical benefit (Felip *et al*., 2010; Strauss *et al*., 2008). The 2011 IASLC/ATS/ERS classification identified patients in the high-risk group of recurrence such as those with micropapillary and solid predominant tumors. Additionally, the prognostic factors recently identified by other groups, such as nuclear grade, cribriform pattern, TTF-1 negativity, high Ki-67 labeling index, immune markers, and SUVmax on FDG-PET, provided better prognostic stratification than the 2011 IASLC/ATS/ERS classification alone (Kadota *et al*., 2012a; Kadota *et al*., 2012b; Kadota *et al*., 2013a; Kadota *et al*., 2013b; Nitadori *et al*., 2013; Suzuki *et al*., 2013). Therefore, we believe that the new classification, which includes the previously mentioned factors, is crucial for identification of stage I lung adenocarcinoma patients at a high-risk of recurrence who may benefit from adjuvant chemotherapy, thus improving their overall survival rate.

The application of the new classification system in small specimens, including cytology, is challenging and requires further investigation. In those small specimens, there may be other morphologic findings, such as nuclear grade (nuclear atypia and diameter), which could help stratify patients based on their risk of recurrence or cancer-related death (Kadota *et al*., 2012b; Sigel *et al*., 2012).

Although the prognostic values of the 2011 IASLC/ATS/ERS classification have been validated, reproducibility (interobserver agreement) has not been adequately investigated to identify a predominant pattern

in lung adenocarcinomas. To confirm reproducibility and improve identification of each histologic pattern using the new classification system, development of precise definitions combined with better training in the interpretation of the system's terminology is necessary.

**Indian context**

With the number of lung cancer diagnoses increasing and the need for management of cancer growing in India, this new classification system is both timely and much needed. The new IASLC/ATS/ERS classification, while requiring a certain amount of additional training for pathologists interested in lung cancer, can be implemented early in hospitals performing H&E staining. Furthermore, the H&E slide can be easily re-reviewed by pathologists at other treatment centres to confirm the diagnosis. Unlike molecular testing, perceived as complex with requirement of resources and advanced equipment, the IASLC/ATS/ERS classification system is easy to implement with low maintenance costs. Awareness of this new classification system and appropriate collaboration with high-volume centers for validation of the predominant histological subtype on H&E slides will assist treating physicians in stratifying prognosis of their patients. There is the possibility that this new classification system may identify differences in Indian patient's histologic subtypes which will form the basis for a modified classification for lung cancer management in Indian patients. However, in order to apply the new classification system to clinical practice in India, prognostic value of histologic subtypes should be confirmed and validated using cohorts of Indian patients.

**SUMMARY**

The 2011 IASLC/ATS/ERS classification system has been proven to have powerful prognostic value in four large cohorts (> 300 patients) across multiple countries (Jokoji *et al*., 2010; Rodriguez *et al*., 2013; Thunnissen *et al*., 2012; Yoshizawa *et al*., 2011). Patients with AIS and MIA show 100% DFS with no recurrent diseases. Patients with micropapillary or solid predominant tumors are classified as a high-risk group for recurrence or cancer-related death. Patients with acinar predominant tumors will be classified as an intermediate-risk group. Patients with papillary predominant tumors may be classified as an intermediate-risk group, although further investigation will be needed. On the basis of our published studies, additional prognostic factors (nuclear grade, cribriform pattern, high Ki-67 labeling index, TTF-1 negativity, immune markers and SUVmax on FDG-PET), combined with the 2011 IASLC/ATS/ERS classification, will further stratify patients into prognostic subgroups for recurrence and cancer-related deaths. Ultimately, this may aid in clinical management and decision making, particularly for patients with early-stage lung adenocarcinomas, and provide informed decision-making as to the option of adjuvant chemotherapy.

To emphasize that the new classification

system of lung adenocarcinomas, in the predominant type of lung cancer observed in India, is readily implementable at any hospital in the country with a pathology laboratory handling routine H&E staining. The reproducibility of the classification system and the prognostic importance for patients with lung cancer in this setting requires further investigation.

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**CONFLICT OF INTEREST**

The authors claim no conflict of interest.

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# Visual Inspection with Acetic Acid as a Screening Test for Cervical Cancer

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Visual inspection with acetic acid (VIA) has been extensively investigated and accepted as potential alternative to cytology or Human Papilloma Virus (HPV) screening in limited resource settings. In developing countries, VIA may have several advantages over cytology or HPV screening. The consumables of the test are low-cost and readily available. VIA has potential of achieving large population coverage, as the test can be performed by a wide range of trained health care personnel and requires basic health infrastructure. It is a real-time test and offers logistic advantage of providing treatment for screen positive women during the same visit leading to high treatment coverage. The sensitivity and specificity estimates of VIA generally fall within the range of those reported for cytology and HPV testing. Randomized controlled trials evaluating test performance of VIA have demonstrated reduction in cervical cancer incidence and mortality in study population. The major limitation of VIA is that it is a subjective test and accuracy is dependent on the skill of trained providers. Low specificity and sub-optimal positive predictive value results in unnecessary referrals and/or treatment which can offset the perceived low cost of the test. VIA based screening programs are required to have clearly defined measurable indicators and a framework to identify the program strengths and weaknesses. Quality assurance of VIA is challenging specially because there is limited information on the test performance in multi-provider real programmatic setting. High quality training, periodic refresher courses, expertise of trained providers and close monitoring of performance indicators are required to ensure good quality VIA.

## INTRODUCTION

### Visual screening for cervical neoplasia

Visual screening for cervical neoplasia includes visual inspection with acetic acid (VIA) and visual inspection with Lugol's iodine (VILI). VIA involves naked eye examination of the uterine cervix under bright light (preferably a halogen focus lamp) one minute after application of 5% dilute acetic acid. Use of magnification does not improve the performance of VIA (Sankaranarayanan, 2004b). VIA is currently being used as a screening test for the national cervical cancer screening programs of many low and medium resource countries in South Asia like Bangladesh (Ahmed, 2008). Visual screening tests are simple, widely feasible and affordable. The test provides immediate results enabling diagnosis and/or treatment to be carried out in the same session for screen positive women. They can be provided by a wide range of health professionals including

**Key words**: Cervical cancer screening, Cervical neoplasia, Visual Inspection with Acetic Acid (VIA), VIA accuracy, VIA limitations, VIA performance indicators, VIA quality control, VIA provider performance.
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doctors, nurses, midwives and primary health care workers after a short period of training. The infrastructural need is minimal and the consumables are universally available.

**Accuracy of VIA: range in sensitivity and specificity**

A systematic review of the accuracy of conventional cytology reported that, in 12 studies with the least biased estimates, sensitivity ranged from 30–87% and specificity from 86–100% (Nanda, 2000). A critical review by Pan American Health Organization (PAHO) in 2003 observed the sensitivity of VIA to range from 29–95.8% and specificity to range from 64.1–97.7% with CIN 2 as the threshold for positive diagnosis (PAHO, 2003). Most of these studies had verification bias since all the women with negative test were not evaluated by the reference standard. International Agency for Research on Cancer (IARC) conducted cross-sectional studies involving 56,939 women aged 25–65 years in Burkina Faso, Congo, Guinea, India, Mali and Niger to evaluate the accuracy of VIA performed by health workers (Sankaranarayanan, 2004a). In all these studies same definitions were used to characterize the test outcomes and all the VIA negative women had gold standard test of Colposcopy to avoid verification bias. The pooled sensitivity, specificity, positive and negative predictive values for VIA in these multi-centric studies were 76.8% (95% CI: 74.2–79.4%), 85.5% (95% CI: 85.2–85.8%), 9.4% (95% CI: 8.8–10.8%) and 99.5% (95% CI: 99.4–99.6%), respectively. The accuracy of VIA was similar or higher than that of Pap smear in such studies where both visual tests and cytology were concurrently evaluated.

**Comparative efficacy of VIA with cytology and HPV DNA testing**

Randomized Controlled Trials (RCT) provides realistic evidences on efficacy of various cervical cancer screening tests and the resulting impact they have on reduction of disease burden. In India a large cluster randomized trial (Sankaranarayanan, 2009) investigated the effect of single round of screening with VIA, cytology and HPV DNA tests among women aged 30-59 years. The study demonstrated that a single round of HPV testing was associated with a significant reduction in number of advanced cervical cancer cases and deaths as compared to the unscreened control group. No such effect was seen in either the VIA or cytology screened group. The findings of the trial indicated that HPV testing could be used as a primary screening technique in low resource settings as it is less demanding in terms of training and quality assurance. Presently, the major drawback is the high cost involved in implementing such a program.

In another cluster RCT (Sankaranarayanan, 2007) in South India, more than 49,000 women aged 30–59 years were followed for 7 years after a single round of VIA screening. The study observed an overall 25% reduction in cervical cancer incidence and 35% reduction in mortality in the intervention group as compared to the unscreened control group. Furthermore, the

maximum benefit of VIA screening was observed in the 30–39 age groups with 38% reduction in cervical cancer incidence and 66% reduction in cervical cancer mortality. The study concluded that in the presence of good training and sustained quality assurance, VIA is an effective method to prevent cervical cancer in developing countries.

The safety and efficacy of 'screen and treat' approach to prevent cervical cancer were evaluated in an RCT (Denny, 2005) at South Africa in which the screen positive women were treated by cryotherapy without resorting to colposcopy. The study demonstrated that 'screen and treat' approach of treating the HPV positive women with cryotherapy was superior to the immediate treatment of the VIA positive women. Treatment of the HPV positive women resulted in 77% lower prevalence of CIN 2+ in subsequent follow up as compared to 37% lower prevalence of CIN 2+ in the women treated for positive VIA. This landmark trial showed that treating women with positive HPV DNA test or VIA with cryotherapy is safe and has a significant impact on the prevalence of CIN 2+ among women participating in such a program.

**Limitations of VIA**

The visual tests are subjective in nature and provider dependent, resulting in a wide variation of performance in different settings. Quality assurance for visual screening is a challenging task specially because there is limited information on the test performance in multi-provider real programmatic setting. VIA is dependent on the full visibility of the transformation zone of the cervix. However, the transformation zone moves into the endocervical canal after menopause and may be totally invisible with aging, limiting the utility of visual tests in postmenopausal women. Moreover, the interpretation of VIA is difficult in postmenopausal women due to the atrophy of the cervical epithelium. Reasons for false positive and false negative results of VIA are summarized in Table 1. Low specificity and suboptimal positive predictive value of VIA will result in unnecessary referrals and/or treatment which can offset the perceived low cost of the tests. There are several training issues related to VIA such as the duration of training, structure and content of the competency based course and post-course evaluation need to be standardized.

**VIA TEST PROCEDURES**

**Pre-test counseling**

The test procedure and the implications of the test results are explained to the woman before proceeding to examine her. She is assured that the discomfort associated with the procedure is minimal. Adequate privacy should be maintained in the examination room. Such measures will ensure that the woman is fully relaxed during pelvic examination. Some programs may require the woman to sign an informed consent prior to the procedure.

**Steps of VIA**

The woman is made to lie in lithotomy position or dorsal supine position with legs flexed. A good focusing light, preferably one containing a halogen bulb should be used to

**Table 1: Reasons for false positive and false negative result of VIA**

| False positive | False negative |
|---|---|
| Immature squamous metaplasia | Inability to identify acetowhite area |
| HPV infection | Inability to categorize acetowhite area |
| Blanching of columnar epithelium | Endocervical lesion |
| Inflammation | |
| Post-menopausal atrophy | |

visualize the genitalia and cervix. The external genitalia are inspected for any excoriation, skin changes, ulceration, wart or growth before proceeding to insert the lubricated self-retaining bivalve speculum. The cervix is gently exposed and any evidence of infection such as purulent, curdy white or greenish yellow or malodorous discharge, vesicles, redness and inflammation are investigated. The cervical os and the squamo-columnar junction (SCJ) are identified. If there is any cervical polyp or ulcer or growth it should be noted. A cotton swab is used to apply 5% freshly prepared acetic acid generously on the entire cervix for one minute before the acetowhite changes are looked for. If there is any discharge or mucus on cervix, it is gently removed while applying the acetic acid. The cervix is examined for any well-defined, well-demarcated, opaque acetowhite areas abutting the SCJ or the external os or extending into the endocervical canal. After the completion of examination any acetic acid collected in the posterior vaginal fornix should be removed with a dry swab. The speculum should be gently withdrawn. The test findings should be explained to the woman and she should be appropriately advised if any abnormality is detected. Meticulous attention should be given to ensure infection control.

**Reporting VIA test results**

VIA test results are interpreted one minute after application of acetic acid. Acetowhitening is not specific to neoplasia. It can be associated with immature metaplasia, inflammation, regenerating epithelium and HPV infection. Acetowhitening associated with cervical neoplasia are localised in the transformation zone of the cervix, invariably arising from the SCJ, have a smooth well demarcated margin and are densely white.

Different authors have used different criteria to define the VIA outcome. In the present document we have used the test definitions as per the technical manual by IARC where VIA is reported as negative, positive or invasive cancer (Sankaranarayanan, 2003). A negative report implies that screening test is normal and no further evaluation is necessary. The woman should be referred for Colposcopy if VIA is reported positive. The suspected invasive cancers on VIA may be directly referred for diagnosis and treatment.

***VIA test definitions as per criteria set by IARC, Lyon***

VIA is reported negative when any of the following features are seen:

- No acetowhite lesions on the cervix
- Thin transparent acetowhite lesions or

faint patchy lesions or lesions without definite margins
- Polyp protruding from the os taking up acetowhite
- Nabothian cysts taking up acetowhite and appearing as whitish acne
- Faint line-like acetowhitening at the junction of columnar and squamous epithelium
- Acetowhite lesions away from the transformation zone
- Streak-like acetowhitening
- Dot like areas in the endocervix, which are due to grape-like columnar epithelium transiently staining with acetic acid

VIA is reported positive when any of the following features are seen:
- Distinct, well defined, dense, opaque or dull white or oyster white acetowhite areas touching the SCJ or touching the external os (if SCJ not seen)
- The lesion with a well-defined margin may or may not be raised from the surface

VIA is reported suspicious of invasive cancer when any of the following features are seen:
- Visible growth or ulcer on the cervix that bleeds on touch
- The growth or ulcer may or may not be acetowhite after acetic acid application

## QUALITY CONTROL AND QUALITY ASSURANCE FOR VIA

### Principles of quality control for cervical cancer screening program

To make the cervical cancer screening program efficient and cost-effective appropriate monitoring and periodic performance evaluation should be done. The ultimate 'impact' of cervical cancer screening program is the reduction of incidence of cervical cancer and mortality from the disease. Initially the program is likely to detect many of the undiagnosed prevalent cancers that may be reflected as an apparent increase in the incidence. There will be a stage-shift of the detected invasive cancers with more cases being diagnosed at earlier stages. As the cervical precancers are detected and treated, there will be a gradual reduction in new cases of invasive disease. However, reduction in incidence and mortality as an impact of screening program may take a decade to be evident. In the meantime, evaluation of performance of the program can be done by assessing the following performance indicators:
- Coverage of the target population
- VIA positivity and positive predictive value
- System capacity (time to colposcopy, compliance to colposcopy, etc.)
- Colposcopy performance (i.e., biopsy rate, colposcopy-histology agreement)
- Pre-cancer detection rate
- Disease extent at diagnosis
- Treatment performance (compliance to treatment, cure rate after treatment)

### Objectives of quality control for VIA

VIA being an observer dependent test requires stringent quality control for optimum performance. The quality standards and the

performance indicators should take into consideration all the components of a VIA-based screening program rather than the test in isolation. The Quality Control and Quality Assurance document for a VIA-based screening program should:

- Clearly define the measurable indicators that will help assess the performance of the program in achieving the stated targets and goals
- Provide a framework to identify the strengths and weaknesses of the ongoing program as well as report and resolve problems at the earliest
- Help continuous improvement in quality for all aspects of cervical screening service delivery

The performance indicators should cover all levels of services – public education and outreach, screening facilities, colposcopy and treatment facilities, pathology laboratories and training program. Some of the quality standards may not be universal and may vary from one programmatic setting to another. Over time, with regular monitoring and reporting of the various performance indicators, an evidence base will generate that will permit the setting of targets for individual program. Data obtained from a well-designed pilot study prior to launching a population based screening program can serve as quality standards for future evaluation of the program.

**Performance indicators for VIA based screening program**

***Coverage of the eligible population***

- Definition: Percentage of eligible women in the target population with at least one VIA test in a three to five years period depending on the specified screening interval
- Method of calculation: (Number of women who have had VIA in last N years ÷ Number of eligible women) × 100
  N = Specified screening interval in the program

Explanation: Ensuring the participation of majority of eligible women in the screening program is one of the key determinants of success of the program. The age at which screening will be initiated and the age at which screening will be discontinued need to be predetermined depending on the capacity and the resources available. Similarly, the interval between two rounds of screening may vary from program to program. The program manager should ensure that all women within the specified age group have access to VIA and a majority undergo the test on regular basis. In an opportunistic program with low participation rate, usually the low risk women undergo frequent rounds of screening, while those with significantly higher risk are left out. For significant reduction of mortality from cervical cancer, 70-80% eligible women should have regular cervical screening.

***VIA test positivity***

- Definition: Percentage of women reported positive/invasive cancer on VIA.
- Method of calculation: (Number of women reported positive/invasive cancer on VIA ÷ Number of women screened) × 100

- Explanation: The positivity of VIA depends on the age distribution of the screened women, prevalence of cervical neoplasia in the target population, skill and experience of the VIA providers. The test positivity will be high in younger women, especially those below 30 years due to the metaplastic changes in the cervix and high prevalence of low grade intraepithelial lesions. In various studies it has been observed that the test providers tend to report higher positivity initially. As they acquire skill and gain confidence, the test positivity tends to come down and stabilizes at a rate appropriate for the population. The optimum VIA test positivity is 5–10% in women between 30–60 years of age. The providers need retraining if the test positivity becomes too low (possibility of missing disease) or too high (possibility of high false positives). If possible, the test positivity should be calculated by 10-year age groups.

***Compliance to colposcopy***

- Definition: Percentage of VIA positive women undergoing colposcopy following a positive VIA test.
- Method of calculation: (Number of VIA positive women who had colposcopy within N months ÷ Number of Women reported positive on VIA in a 12 month period) × 100

  N = 1–3 months of index VIA test, depending on the program capacity and specification
- Explanation: Ideally all screen positive women should have colposcopy for confirmation of the disease status that will lead to appropriate treatment. Linkage between screening and colposcopy/treatment is essential for the success of the screening program. A major advantage of VIA is that the report is immediately available and the positive women can have colposcopy in the same sitting or can be advised for colposcopy immediately. The program managers will have to decide on the permissible interval between VIA test and colposcopy depending on the program capacity. More than 80% of the VIA positive women should have colposcopy within the specified time.

***Biopsy rate and adequacy of biopsy specimens***

- Definition: Percentage of VIA positive women who received a histological diagnosis in a 12 month period is defined as the biopsy rate. The proportion of all the biopsies obtained during colposcopy that are reported unsatisfactory by the histopathologist is the measure of inadequate biopsies.
- Method of calculating biopsy rate: (Number of women with a histological diagnosis after VIA ÷ Number of VIA positive women in a 12 month period) × 100
- Method of calculating inadequate biopsy rate: (Number of women with a diagnosis of inadequate biopsy ÷ Number of VIA positive women in a 12 month period)

× 100

- Explanation: Punch biopsies are obtained from cervix if cervical neoplasias are suspected during colposcopy of the VIA-positive women. Biopsy is obtained through loop excision if 'see and treat' policy is practiced during colposcopy. A low biopsy rate usually indicates poor predictive value of VIA or inadequate follow up. The biopsy rate also depends on the skill and thoroughness of the colposcopist to rule out neoplasias. Sometimes the women themselves refuse biopsy. The minimum biopsy rate acceptable as a performance measure is to be specified in individual program setting and to be monitored over time. High inadequate biopsy rate indicates use of inappropriate punch biopsy forceps or inadequate skill of colposcopist to obtain a good biopsy. Failure to preserve the specimen or to process the specimen correctly in the laboratory can also lead to a report of unsatisfactory biopsy. The rate of inadequate biopsies should be as low as possible and corrective measures should be taken promptly if a high rate is detected.

***Detection rate for cervical cancer precursors***

- Definition: Number of pre-cancerous lesions detected per 1,000 women who had a VIA test in a 12 month period. Commonly the detection rate of CIN 2 and CIN 3 are calculated due to the clinical and programmatic relevance of the high grade precursor lesions.
- Method of Calculation: (Number of women with CIN 2 and CIN 3 on histology ÷ Number of women who had VIA in a 12 month period) × 1000
- Explanation: Detection rate of CIN, especially CIN 2 and CIN 3 lesions, can serve as a surrogate for the sensitivity estimate of VIA test. The detection rate of CIN 2+ lesions in the population also depends on the prevalence of the disease in the population and capability of the colposcopist to identify the disease correctly. The detection rate has to be monitored over time. A decline in the rate indicates suboptimal sensitivity of VIA or inadequate work up of the VIA positive women. The detection rate of CIN 2 and CIN 3 usually varies from 3–10 per 1000 screened population.

***Stage of invasive cancer at diagnosis***

- Definition: Proportion of screen detected invasive cancers in early stage (stage I; confined to cervix)
- Explanation: One of the major indicators of effectiveness of screening program is the ability to detect cervical cancers in the preclinical or early stage when the cancer is curable in more than 90% cases. In an unscreened population the majority of cancers are detected in advanced stage. With implementation of screening program the number should come down gradually.
- Method of calculation: (Number of cancers of cervix in stage I ÷ Number of

cancers detected in a 12 month period) × 100

***Incidence of cervical cancer***

- Definition: Age standardized incidence of cervical cancer in the screened population.
- Explanation: As organized screening program become established the rates of cervical cancers come down eventually due to intervention at the precancer stage of the disease. The incidence rate is recorded by a population based cancer registry operating among the screened women and is expressed as age-standardized rate. A reduction in incidence (and mortality) rate of cervical cancer is the best outcome indicator for cervical cancer screening program but may not be feasible to obtain in many of the low/medium resource settings due to non-availability of population based cancer registry.
- Method of calculation: To calculate age standardized incidence rate, information regarding the number of cancers detected in one year, their age distribution and the age distribution of a standard population is necessary.

Appropriate infection control and sterilization procedures should be practiced and should be monitored as part of standard quality control measure.

In addition, following practical issues also influence accuracy of the test:

- Use of correct concentration of acetic acid (5%)
- Use of a good quality light source, preferably a halogen lamp
- Use of a stop-watch to monitor the time (one minute) required for interpretation of test following application of acetic acid

**Individual VIA provider performance evaluation**

VIA is an observer dependent test. For correct interpretation of the post acetic acid application changes, appropriate training of the test providers is essential. VIA training manuals developed by IARC (Sankaranarayanan, 2003) or JHPIEGO (McIntosh, 2001) may be used. The duration of training varies between 5–10 days (JHPIEGO, 2001a; Blumenthal, 2005), during which the candidates should have exposure to adequate number of VIAs being performed by the trainer as well as by the trainee. The minimum number of VIAs required to be observed and to be performed under supervision to gain adequate competency to perform the procedure independently is not yet standardized. It is generally agreed that the number should be 50–100, of which at least half of the procedures should be done by the trainee.

It is essential that after completion of training each trainee should undergo competency based evaluation. During such evaluation the trainee should perform adequate number of VIAs while being observed by a trainer. The trainer has to evaluate the trainee using a checklist that can assess the trainee's skill in counseling (before and after VIA), positioning of the woman, steps of VIA, interpretation of the appearance

of the cervix before and after application of acetic acid and following appropriate infection control measures. All VIA providers need a reorientation training at least once a year. The agreement between the VIA results obtained by the provider and those obtained by the trainer can be assessed during such reorientation training. Such agreement should be at least 80%.

## CONCLUSION

For implementing successful cervical cancer screening programs, it is essential to have sufficient resources to cover the entire target population and provide diagnostic as well as treatment facilities to all women identified as positive by screening tests. Evidences from various studies suggest use of HPV DNA testing as a primary screening tool as it is objective, reproducible, highly sensitive and demonstrates a high negative predictive value. Due to logistic and fiscal constraints many of the low and medium resource countries have not been able to introduce HPV based screening program.

On the other hand VIA is feasible, effective and an inexpensive alternative to cytology or HPV DNA based screening programs in countries with limited resources. Moreover, it offers logistic advantage in providing diagnosis and/or treatment for screen positive women during the same visit leading to high treatment coverage. VIA quality control is challenging but when performed by well trained and experienced providers under good monitoring and supervision, it is an effective alternative screening method for prevention of cervical cancer in a low resource setting.

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## CONFLICT OF INTEREST

The authors claim no conflict of interest.

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# Ovarian Cancer: An Ever Challenging Malady

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Ovarian cancer is the fifth leading cause of cancer related deaths in women with a five year survival rate of only 30–40%. Amongst the three broad subgroups of ovarian cancer, epithelial ovarian cancer is the most common and is divided in mainly five subtypes based histology and clinical behaviour. In patients when the disease is still confined to ovaries, surgery alone is curative for more than 90% patients. Unfortunately, most women are diagnosed with advanced stage disease and recurs in majority despite of debulking surgery and initial response to chemotherapy. Thus ovarian cancer is still a challenge to clinicians which gets more complicated due to asymptomatic nature of the early stage disease and frequent development of resistance to standard therapies. Therefore, researchers worldwide are engaged in identifying markers for early detection of ovarian cancer, investigating molecular mechanisms of chemoresistance, improving detection methods and developing novel therapeutic measures. In this review, we attempt to discuss the contemporary research and challenges associated with epithelial ovarian cancer along with the future improvements in various areas such as early detection of ovarian cancer through Multiplex-Methylation specific PCR (MSP) assay and Serial Analysis of Gene expression (SAGE) assay and identifying new biomarkers, facilitating personalised chemotherapy regime by various chemo-response assays, novel drugs and targeted therapies which will aid in enhancing the overall survival rate in future and overcome this deadly gynaecologic disease.

## INTRODUCTION

Ovarian cancer is a lethal cancer amongst the gynaecologic malignancies. Approximately 239,000 new cases are reported worldwide annually and around 152,000 women succumb to this fatal disease annually (GLOBOCAN, 2012). In India, ovarian cancer is the fourth most common cancer in women with an annual occurrence of 26,834 new cases (GLOBOCAN, 2012). Although majority of ovarian cancer incidence occur in post-menopausal women of 60–64 years, young women below the age of 20 often experience germ cell tumors, while borderline tumors are often presented in women in the median age of 30–40 years (Berek *et al.*, 2012). A higher incidence of ovarian cancer has been recorded in women with reproductive risk factors such as nulliparity, history of infertility, early menarche and late menopause. Multiparity and use of hormonal contraceptives are thought to act as a parapet against ovarian cancer (Negri *et al.*, 1991; Berek *et al.*, 2012).

At early stages, ovarian cancer is highly

**Key words:** Ovarian cancer, early detection, chemoresistance, chemoresponse assays, chemotherapeutic drugs.
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asymptomatic and therefore, remains undetected. Elevation of Cancer Antigen-125 (CA-125) level in blood and ultrasonography help to confirm presence of ovarian cancer (Rauh-Hain *et al.*, 2011). A combination of cytoreductive surgery and platinum based chemotherapy are used to thwart growth of tumor (Xiao *et al.*, 2012). However, often patients succumb to ovarian cancer due to recurrence of the disease (Perez *et al.*, 1993).

The significantly high relapse of ovarian cancer is attributed to acquirement of chemoresistance, thus preventing total elimination of ovarian cancer cells. Development of chemoresistance in cancerous cells is complex, and occurs due to several reasons including expression of beta-tubulin isotypes, over expression of P-glycoprotein (PGP) mediated expulsion of chemotherapeutic drugs, altered DNA repair mechanisms, increased drug detoxification, increased cell survival and decreased apoptosis (Gaikwad *et al.*, 2012; Ling, 2005). Chemoresistance acquired by tumor cells decreases the success of overcoming complete cure in ovarian cancer patients. Demonstration of differential chemoresponses indicates the need for personalized treatment regimens.

In the current review, we will highlight commonly used tumor markers and novel approaches towards early detection of ovarian cancer, multifactorial causes of chemoresistance, exploratory research towards development of chemoresponse assays and drugs currently in clinical trials to treat ovarian cancer efficaciously.

**Ovarian cancer: A heterogeneous disease**

The biggest challenge associated with ovarian cancer treatment is the enormous heterogeneity. The World Health Organization classification of ovarian tumors based on tissue of origin are as follows: surface epithelial-stromal tumors (65–70%), germ cell tumors (15–20%), sex cord stromal tumors (5–10%) and metastatic tumors (5%) (Berek *et al.*, 2010; Lee-Jones, 2004; Scully, 1987). Earlier notion of classifying serous (85%) (low and high grade), endometrioid (10–20%), mucinous (3–5%), clear cell (5–10%), Brenner tumors, transitional tumors and undifferentiated (< 1%) tumors as epithelial ovarian tumors is recently debated (Berek *et al.*, 2012; Lalwani *et al*., 2011; Kumran *et al.*, 2010). Since these subtypes show widely different clinicopathological features and behaviour, current classification categorizes ovarian cancer in two groups of Type I and Type II. Tumors that originate from epithelial lining of the ovary are clinically indolent and classified as Type I (includes low-grade micropapillary serous carcinoma, low-grade endometrioid, clear cell and mucinous carcinomas). Type I tumors grow slowly, usually from borderline tumors, present at stage 1a and show mutations in several oncogenes like *kras, braf, pten, arid1a, ppp2r1a* and *ctnnb1*. Tumors that are probably non-ovarian in origin but migrate to ovary often arise from the epithelium of fallopian tubes or through endometriosis and are grouped as Type II (includes high-grade serous carcinoma, high-grade endometrioid carcinoma, malignant mixed mesodermal

tumors and undifferentiated carcinomas). Type II tumors are present at advanced stages III and IV, aggressive in nature, exhibit mutations in *p53*, *brca1* and *brca2* (Kurman *et al.*, 2008). Type I tumors comprise of 20–30% of Epithelial Ovarian Cancer (EOC) (Bast *et al.*, 2009) while Type II tumors account for 70–80% cases (Colombo *et al.*, 2013).

Besides the histogenetic groups of ovarian tumors, the International Federation of Gynecology and Obstetrics (FIGO) have classified ovarian cancer in following stages:

**Stage I: Growth limited to ovaries**

IA - Growth limited to one ovary; no ascites present containing malignant cells. No tumor on the external surface; capsule intact

IB - Growth limited to both ovaries; no ascites present containing malignant cells. No tumor on the external surface; capsule intact

IC* - Tumor either stage IA or IB, but with tumor on surface of one or both ovaries, or with ascites present containing malignant cells, or with positive peritoneal washings

**Stage II: Growth involving one or both ovaries with pelvic extension**

IIA - Extension and/or metastases to the uterus and/or tubes

IIB - Extension to other pelvic tissues

IIC - Tumor either stage IIA or IIB, but with tumor on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings.

**Stage III: Tumor involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis. Superficial liver metastases equals stage III**

IIIA - Tumor limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologically proven extension to small bowel or mesentery.

IIIB - Tumor of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative.

IIIC - Peritoneal metastasis beyond the pelvis > 2 cm in diameter and/or positive regional lymph nodes.

**Stage IV: Growth involving one or both ovaries with distant metastases**

If pleural effusion is present, there must be positive cytology to allot a case to stage IV. Parenchymal liver metastasis equals stage IV.

*In order to evaluate the impact on prognosis of the different criteria for allotting cases to Stage IC or IIC, it would be of value to know if rupture of the capsule was spontaneous, or caused by the surgeon; and if the source of malignant cells detected was peritoneal washings, or ascites (Heintz *et al.*, 2006).

**Early detection of Ovarian Cancer**

A major hurdle associated with effective treatment of ovarian cancer is "Early Detection". A majority of women exhibit vague symptoms like altered bowel and bladder habits, abdominal pain and swelling,

dyspepsia, nausea, vomiting, unusual fatigue and weight changes that are often misinterpreted as normal changes during menopause or ageing, and are often not correlated to the presence of ovarian cancer (Bankhead *et al.*, 2005). Therefore, ovarian cancer remains asymptomatic in early stage and is frequently detected at advanced stages, III or IV (Lalwani *et al.*, 2011; Sankaranarayanan *et al.*, 2006). Hence, it is pertinent to detect ovarian cancer at an early stage in order to treat patients effectively and increase survival.

Standard ways of detecting ovarian cancer include: ELISA-based approach to identify tumor markers, transvaginal ultrasound, magnetic resonance imaging (MRI), and computed tomography scan (CT).

**Tumor markers for detecting ovarian cancer**

Unlike cervical cancer where detection of high risk human papilloma viruses and a Pap smear test screens for presence of malignancy, ovarian cancer lacks defined screening tests. Thus, there is a need for novel molecular approaches to detect ovarian cancer at early stages. Biomarkers are unique biomolecules found in bodily fluids like blood, urine, serum, as well as in tissues, that may directly correlate with the presence of malignant tumors (Husseinzadeh, 2011).

A specific glycoprotein, CA-125 or MUC16, is currently used in clinics as a biomarker to detect disease and examine success of chemotherapy in ovarian cancer patients. Although 60% cases of early stage ovarian cancer demonstrate an increase in CA-125, elevated levels are also seen in cancers of fallopian tube, endometrium, breast and lung. Hence, CA-125 is not highly specific to ovarian cancers (Husseinzadeh, 2011). Besides, CA-125 may also be elevated in many benign conditions such as endometriosis, tuberculosis, fibroids, pelvic inflammatory disease. Although, CA-125 is neither sensitive nor specific for ovarian malignancy, however, currently it is the only serum marker widely used for early detection of the disease.

Recently, it has been demonstrated that secreted glycoprotein human epididymis protein 4 (HE4) is expressed at higher levels by serous and endometrioid epithelial ovarian cancer cells and may be used as a candidate tumor marker for these tumors (Drapkin *et al.*, 2005). HE4 and CA-125 tests along with the menopausal status of the woman is used in calculating the risk of ovarian cancer, using the risk of ovarian malignancy algorithm (ROMA), often used as a supplement to the standard pre-surgical evaluation of an adnexal mass to further assess the likelihood of malignancy. In September 2011, the US Food and Drugs Administration (FDA) approved the use of HE4 in calculation for ROMA.

Consistent efforts to identify new and alternative markers for ovarian cancer are ongoing. However, sensitivity and specificity remain a challenge. A study by van Haaften-Day and colleagues showed a combination of biomarkers CA-125, OVX1, and M-CSF (Macrophage-Colony Stimulating Factor) enabled detection of 85% of the ovarian cancer, while CA-125 alone could identify

only 69% of the cancers (van Haaften-Day *et al.*, 2001). Another study demonstrated elevated mesothelin in urine in 42% and 75% of early stage and advanced stage ovarian cancer, respectively (Badgwell *et al.*, 2007), emphasizing further evaluation of urine mesothelin as a potential biomarker for early detection of ovarian cancer. Bikunin, a glycoprotein secreted by hepatocytes that inhibits metastasis may be used as a probable prognostic marker for ovarian cancer. In a pilot study of 327 ovarian cancer patients, Bikunin was elevated in patients with inferior quality of debulking tumor and exhibited poor response to chemotherapy, with a survival period of 26 months (Matsuzaki *et al.*, 2005).

Other tumor markers, such as osteopontin, human kallikreins, M-CSF, vascular endothelial growth factor (VEGF), leptin, prolactin were reported to be associated with ovarian cancer and need further investigation (Husseinzadeh, 2011).

MicroRNAs (miRNAs) are a class of 19–35 nucleotide long post-transcriptional regulators, involved in degradation of messenger RNA (mRNA), and thereby regulate protein translation, as also various physiological processes. These small RNA molecules have emerged as candidate biomarkers for various malignancies (Chen *et al.*, 2013). Numerous studies have reported that anomalous expression of miRNAs in epithelial ovarian cancer may possibly aid detection of ovarian cancer at earlier stages (Chen *et al.*, 2013). Lorio *et al*. (2007) conducted a genome-wide microRNA expression profiling in 15 normal and 69 malignant ovarian tissues. The significant analysis of microarrays (SAM) and partitioning around medoids (PAM) tool analysis, identified 39 miRNAs and 29 miRNAs, respectively, enabling sorting of normal versus tumor samples. The authors further reported four up-regulated miRNAs i.e, *miR-200a, miR-200b, miR-200c, miR-141* and 25 down-regulated miRNAs that include miR-140, miR-145 in ovarian cancers. Further evaluation of these miRNAs in different histological subtypes, demonstrated increased expression of *miR-200a, miR-200c* in serous, endometrioid and clear cell carcinomas; up-regulation of *miR-200b, miR-141* in endometrioid and serous subtypes; increased expression of *miR-203, miR-205, miR-23* in endometrioid type; down regulation of *miR-140, miR-199a, and miR-125b1* in serous, endometrioid, clear cell histotypes, as compared to normal ovarian tissue (Lorio *et al.*, 2007).

However, all these biomarkers have been proven to be suboptimal with limited sensitivity and specificity and high false-negative rate for detection and have not helped to decline mortality due to ovarian cancer. Hence, researchers are looking for novel approaches to detect ovarian cancer at early stages (Zhang *et al.*, 2013) which include MSP and SAGE assays.

**Multiplex Methylation-specific PCR assay**

Methylation of CpG islands in genes can cause deregulated expression, which precedes clinical manifestation of symptoms. In order to identify the status of methylation in circulating

DNA, a novel multiplex methylation-specific PCR (MSP) assay was designed. Caceres *et al.* (2004) used MSP assay on a cohort of 50 patients diagnosed with ovarian tumors or primary peritoneal tumors and 21 archival stage I tumors to analyse the status of hypermethylation of genes *brca1, rassf1a, p14arf, death-associated protein kinase (dapkinase)*. The study reported that 70 out of 71 tumors (37 of 38 stage I tumors and 33 stage III–IV tumors) showed hypermethylation in at least one of the genes (Ibanez de Caceres *et al.*, 2004). Studies have shown anomalous methylation pattern of circulating tumor DNA in serum of patients with tumors of prostrate, colon, lung and breast could be used as prognostic markers (Zhang *et al.*, 2013). Expression of CpG island hypermethylation of seven gene*s – apc*, *rassf1a*, *runx3, cdh1, tfpi2, sfrp5,* and *opcml* was studied in 202 epithelial ovarian cancer serum samples. The multiplex MSP assay has demonstrated 83% specificity, 82% sensitivity and 91% accuracy over CA-125 alone which showed 50%, specificity, 72% sensitivity and 89% accuracy, respectively for early diagnosis of ovarian cancer. Further investigation on status of hypermethylation, hypomethylation, and overall epigenetic changes in genes can lead to better diagnosis of ovarian cancer at earlier stages (Zhang *et al.*, 2013).

**Serial analysis of gene expression assay**

Dr. Victor Velculeses, in 1995, developed serial analysis of gene expression assay (SAGE) to identify specific mRNA transcripts in pathologic state. The assay determines expression of up-regulated or down-regulated genes in neoplasms, and differentiates histological subtypes based on gene expression. *flj12988, cldn3* and *folr1* are some candidate genes which have been identified in ovarian cancer through SAGE assay (Zhang *et al.*, 2011).

**Ultrasonography**

Transvaginal or transabdominal ultrasonography is the standard non-invasive imaging method used in clinic to detect presence of tumors in ovaries (Figure 1a, 1b). Van Nagell *et al*. (2000) analyzed the importance of transvaginal sonography (TVS) in 14,469 asymptomatic women who were either more than 50 years or above 25 years with familial history of ovarian cancer. Two hundred patients who showed absence of abnormality at first TVS were subjected to another scan after a year. While post-menopausal patients presented with tumor volume of more than 10 $cm^3$ and premenopausal patients bearing more than 20 $cm^3$ tumor volume were subjected to another TVS within 4–6 weeks. Finally, 180 patients with repetitive abnormal scans were recommended for surgical debulking of the tumor. Out of 14,289 patients (who initiallyshowed no abnormality on TVS) only four developed ovarian cancer. Thus this study reports TVS screening to have 98% specificity, 81% sensitivity with a positive predictive value (PPV) of 0.094 and a negative predictive value (NPV) of 0.999 (van Nagell *et al.*, 2000). Another study was conducted to assess the efficacy of TVS and CA-125 on a cohort of 312

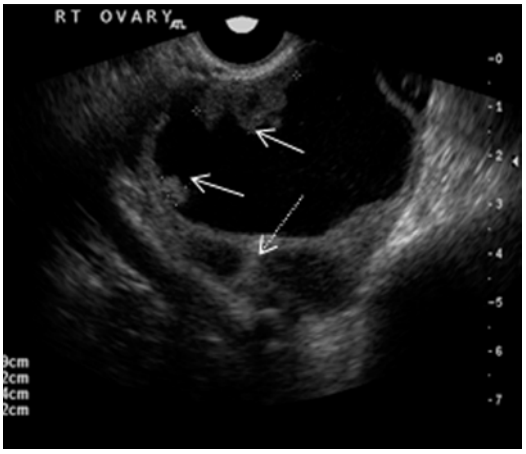


**Figure 1a:** Transvaginal ultrasonography showing a cystic adnexal mass with solid papillary nodules (solid arrows) and thick septations (dotted arrow), suggestive of neoplastic nature.

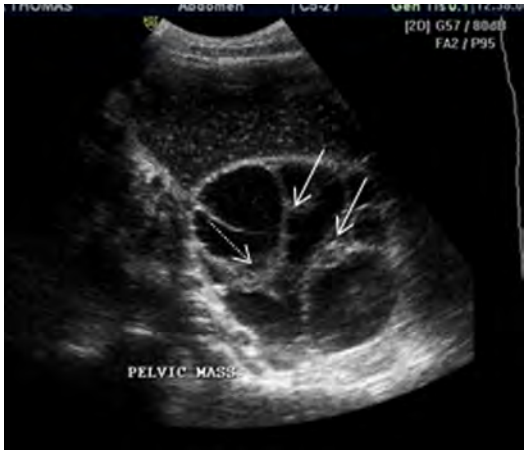


**Figure 1b:** Transabdominal ultrasonography showing a multicystic adnexal mass with thickened walls, thick septations (solid arrows) and solid areas in the centre (dotted arrow).

patients to identify women with high predisposition to ovarian cancer. The study showed TVS alone has a specificity, sensitivity, PPV and NPV of 90%, 40%, 6% and 99%, respectively, and CA-125 alone has a specificity, sensitivity, PPV and NPV of 96%, 60%, 13% and 99%, respectively. A combination of TVS and CA-125 showed better specificity and NPV, each at 99%, and PPV of 40% (Olivier *et al.*, 2006). The data indicated TVS as preferred mode of diagnosis for ovarian cancer despite limitations, which include (1) a 9.3% rate of PPV; (2) inability to differentiate benign from malignant tumors; and (3) ineffective in identifying cancerous cells in normal-sized ovaries (van Nagell *et al.*, 2000). An amalgamation of TVS and serum biomarkers will nonetheless accelerate early-stage detection of ovarian cancer in future (Fishman *et al.*, 2005). Currently, a large clinical trial involving more than 100,000 women is undergoing in UK, to understand the real potential of multimodal screening or MMS (TVS + CA125) against TVS alone. Though not complete yet, this trial indicates higher specificity in the MMS than in the TVS group resulting in lower rates of repeat testing and surgery (Menon *et al.*, 2009).

**Computed tomography (CT)**

Apart from ultrasonography, computed tomography (CT) scans also assist in diagnosis of ovarian cancer (Figure 2). Qayyum *et al.* (2005) have established that CT scans has 96% accuracy in identification of residual cells after surgery (Qayyum *et al.*, 2005). Another study demonstrated that CT scan has 87% precision in detection of benign or malignant tumors along with highspecificity (85%) and sensitivity (90%) and 55% and 89% accuracy in detecting stage I/II and stage III/IV, respectively (Byrom *et al.*, 2002).

**Current treatment modalities**

Advanced ovarian cancer is a Chemoresponsive but often not chemocurable

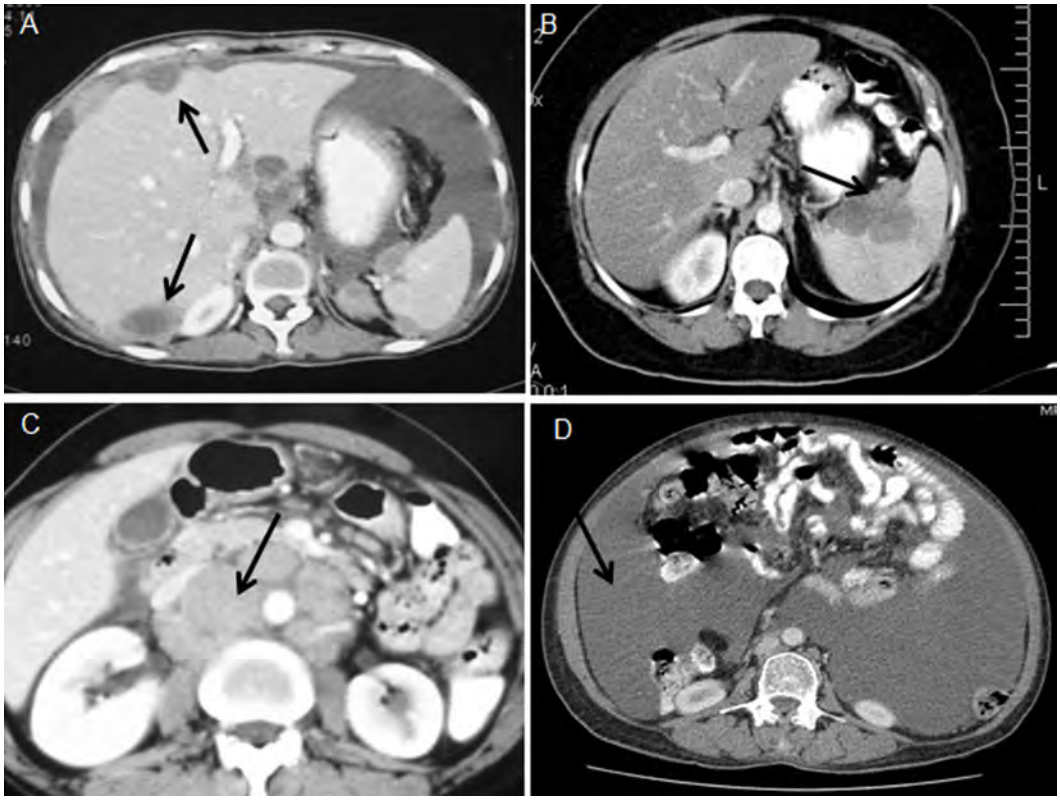


**Figure 2:** Contrast enhanced CT scans in advanced ovarian disease. **A)** Multiple liver surface deposits causing scalloping of the surface; **B)** Splenic hilar deposits; **C)** Multiple enhancing metastatic retroperitoneal nodes; **D)** Severe Ascites.

disease. Chemotherapy administered either intravenously (IV) or intraperitoneally (IP) is platinum-based combination of cisplatin or carboplatin and paclitaxel (Bast, 2011). Cisplatin kills cells by forming inter- and intra-strand DNA adducts via binding to N3 site on purine bases, stalling cell cycle at G2 phase and decreasing the ATP production in mitochondria. While paclitaxel prevents depolymerization of beta-tubulin subunits and blocks cell cycle at metaphase/anaphase of mitosis (Ling, 2005). Other drugs which have shown activity on ovarian tumors are methoxypolyethylene, PEGylated liposomal doxorubicin (PLD), topotecan, etoposide, tamoxifen, methotrexate, gemcitabine, vincristine, vinblastine, docetaxel and vinorelbine (Bookman *et al.*, 2009; Berek *et al.*, 2010).

The cornerstone of ovarian cancer treatment has been surgical removal of tumor followed by adjuvant chemotherapy. Sometimes surgical removal of tumor is difficult due to the extent of the disease. The choice of treatment in such cases is neoadjuvant chemotherapy (NACT) prior to optimal tumor debulking followed with additional chemotherapy (Robinson *et al.*, 2008). Chemotherapeutic drugs are usually administered IV, while ovarian cancer patients who have undergone optimal debulking surgery also have an option of IP

chemotherapy via an IP access port placed at surgery (Robinson *et al.*, 2008). IP chemotherapy has been reported as more than 10-fold effective than IV chemotherapy after surgical debulking and increases overall survival (OS) to 16 months (Bast, 2011). The combinatorial chemotherapy of IV/IP alleviates a median progression-free survival (PFS) of up to 16–21 months and median overall survival from 24–60 months. However, IP therapy remains to be accepted universally due to the adverse side effects like neurotoxicity and increased fatigue. Even with recent advances in treatment modalities, about 60% patients succumb to the disease within five years, which is attributed to relapse and acquired resistance to chemotherapeutic drugs (Armstrong *et al.*, 2006; Bast, 2011; Bookman *et al.*, 2009). Hence, the need of understanding the molecular basis of chemoresistance and relapse is crucial.

**Chemoresistance in ovarian cancer**

Chemoresistance is a phenomenon wherein a patient stops responding to the administered chemotherapeutic drugs, causing aggressive metastases and death (Figure 3). The patient may be intrinsically resistant or may acquire resistance to chemotherapy on successive exposures. Inability to mitigate and counter chemotherapy failure is attributed to several factors as elaborated.

***Aberrant membrane transporters***

Chemotherapeutic drugs are structurally diverse and have dissimilar intracellular targets. The entry–exit in a cell is dependent on transmembrane unidirectional influx and efflux pumps such as ATP-binding cassette (ABC) super-family membrane transporters (Nooter *et al.*, 1991). The ABC super-family membrane transporters consist of 48 genes and are subdivided into eight groups from ABCA to ABCG. The ABC proteins like PGP and multidrug resistance proteins like MDR-associated protein 1, breast cancer resistance protein (BCRP), lung resistance protein (LRP), expedite efflux of chemotherapeutic drugs and hinder accumulation of drugs inside cancer cells (Goff *et al.*, 2001; Ling, 2005).

MDR associated proteins (MRP), first discovered by Cole *et al*. (1992) are transmembrane proteins with a role in the efflux of accumulated drugs from the cells (Goff *et al.*, 2001). There are seven types of MRPs (MRP1MRP7) and each transports drugs in different capacities. MRP1 exhibits poor transport of paclitaxel than drugs conjugated to sulphate, glutathione. Overexpression of MRP2 facilitates removal of cisplatin, etoposide, doxorubicin, epirubicin, mitoxantrone and methotrexate (Borst *et al.*, 2000). MRP3, MRP4, and MRP5 expedite efflux of chemotherapeutic drugs like etoposide and gemcitabine (Hagmann *et al.*, 2010).

Platinum drugs are extremely polar compounds that do not enter a cell through passive diffusion, rather depend on active uptake via membrane associated copper transporters – hCTR1 and hCTR2 (Holzer *et al.*, 2004). Studies in yeast and mammalian cells showed that absence of CTR1 protein hinders platinum containing drug uptake

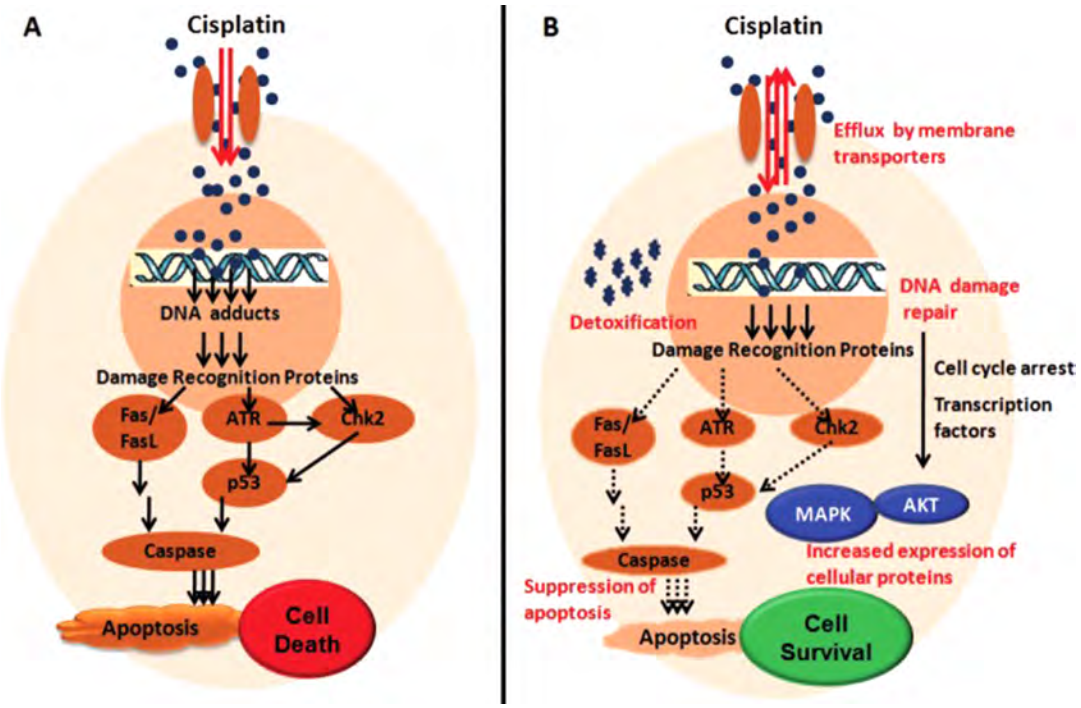


**Figure 3:** Schematic representation of **A:** Mode of action of platinum-based drug cisplatin: Cisplatin enters cell through membrane transporters and forms DNA adducts in the nucleus resulting in activation of DNA damage recognition proteins, which causes cell cycle arrest. Apoptotic machinery is further activated with continuous exposure to cisplatin leading to cell death (arrows in black); **B:** Mechanisms of acquired resistance to cisplatin (text in red): Cisplatin entered in the resistant cells is either **i)** effluxed by up-regulation and overexpression of membrane transporters or **ii)** detoxified by the Glutathione transferases. Formation of lower level of DNA adducts results in the **(iii)** activation of DNA damage repair proteins and further global changes involved **iv)** increased expression of cellular proteins like transcription factors, MAPK and Akt etc., which leads to cell survival and also **v)** suppression of apoptosis eventually leading to chemoresistance. [Source: Gaikwad et al., 2012]

(Holzer *et al.*, 2006; Howell *et al.*, 2010). A study demonstrated that overexpression of hCTR1 in A2780 (ovarian epithelial cancer cell line) not only increased copper influx 13.7 fold but also improved intake of cisplatin by 55% after 24 hours (Holzer *et al.*, 2004). Sensitive ovarian cancer cell lines A2780, 2008 and IGROV-1 were more receptive to cisplatin than cisplatin-resistant A2780, 2008 and IGROV-1 cell lines. These cisplatin-resistant A2780, 2008 and IGROV-1 cells were found to be cross resistant to copper uptake, thus elucidating the role of human copper transporters in influx of platinum drugs apart from copper homeostasis (Katano *et al.*, 2002). Kamazawa *et al*. (2002) analyzed expression of MDR1, MRP1, MRP2 in SKOV-3 (p53-null cells), KOC7c, KF, paclitaxel-resistant KF (KFTx) ovarian carcinoma cell lines and in ovarian cancer patients with relapse after paclitaxel treatment. Increased resistance to paclitaxel and expression of drug resistance genes were noted in SKOV-3, KOC7c, and KFTx cell lines. In addition, 6 of 27 paclitaxel non-responder patients showed increased MDR1 expression (Kamazawa *et*

*al.*, 2002). The study thus emphasized that expression of multidrug resistance genes correlates with higher resistance to paclitaxel.

Anti-oxidant protein 1 (ATOX1) transports circulating platinum drugs to specific organelles and regulates their discharge out of the cell via efflux pumps ATP7A and ATP7B (Howell *et al.*, 2010). ATP7A and ATP7B are P-type ATPase membrane transporters involved in maintaining homeostasis of heavy metals like cadmium, copper, and zinc (Nakayama *et al.*, 2002; Nakayama *et al.*, 2004). ATP7A is present in all the organs except liver, wherein the expression of ATP7B is predominant (Samimi *et al.*, 2004). Katano and colleagues demonstrated increased expression of ATP7A in cisplatin-resistant A2780 and 2008 ovarian cell, and an accrual in ATP7B expression in IGROV-1 cells (Katano *et al.*, 2002). Another study reported a 1.5-fold higher expression of ATP7A in the ovarian cell line, 2008 through transfection with ATP7A expression vector that showed minimal intake of copper and conferred resistance to cisplatin, oxaliplatin and carboplatin (Samimi *et al.*, 2004).

Increased expression of ATP7A was found in 18 of 54 treated ovarian carcinomas with poor survival (Samimi *et al.*, 2003). Expression of ATP7B, MDR1, MRP1, MRP2, LRP and BCRP was analyzed by real-time analysis in 82 ovarian cancer patients exposed to cisplatin-based chemotherapy after tumor debulking. Varied expression of genes [*atp7b* (43.9%), *mdr1* (24.4%), *mrp1* (86.6%), *mrp2* (81.7%), *lrp* (92.7%) and *bcrp* (53.7%)] were noted in the samples with significant expression of *atp7b* ($p$ = 0.01) in relapsed cases, indicating *atp7b* as a strong candidate causing chemoresistance in cisplatin treated and relapsed ovarian cancer patients (Nakayama *et al.*, 2002).

In order to inhibit action of multidrug resistance proteins and achieve better efficacy of cisplatin treatment, several approaches including antisense technology, oligonucleotide combinatorial technology, small molecule inhibitor technology are in use. Several pharmaceutical companies are developing IV agents and oral compounds to block PGP (Persidis, 1999). However, toxicity and undesired inhibition of these transporters in normal organ are often an impediment in the clinical trials.

***Altered drug metabolism***

Another protective mechanism adopted by cells to escape deleterious effects of drugs is the glutathione-dependent detoxification mechanism. Like normal cells, cancer cells try to make drugs ineffective by upregulating the cellular proteins that expedite detoxification. Predominantly glutathione (GSH), glutathione S transferase (GSTs), glutathione peroxidase (GPx) and metallothioneins facilitate detoxification of toxins and drugs, and neutralize reactive oxygen species (Abdullah *et al.*, 2013; Ling, 2005). GSH homeostasis is important as GSH deficiency causes oxidative stress, while excess results in increased antioxidative ability leading to drug inactivity and propelling chemoresistance in tumors (Abdullah *et al.*, 2013; Syng-Ai *et al.*, 2004).

GSTs belong to a family of enzymes that facilitate coupling of glutathione to various molecules, including chemotherapeutic drugs. Functional polymorphism in 3 *gst* genes namely *gstm1, gstt1* and *gstp1* was associated with treatment and survival of a cohort of 215 primary epithelial ovarian cancer patients using PCR techniques such as PCR-RFLP. The study reported an increased progression of the disease in late-stage patients with higher *gstm1* compared to *gstm1* null patients, while no such association of *gstm1* with progression of disease in early-stage patients was noted (Saga *et al.*, 2008). Similarly patients without *gstm1* and decreased *gstp1* polymorphisms had 60% better progression free survival and 40% overall survival than patients with *gstm1* and *gstp1* polymorphisms (Beeghly *et al.*, 2006). Another study reported presence of GPX3 in KK, OVMANA, OVSAYO and RMG-1 (clear cell ovarian carcinoma cell lines) by DNA microarray and real-time PCR. These cells when transfected with shRNA against *GPX3* showed decreased level of *GPX3* expression with increased sensitivity to cisplatin (Saga *et al.*, 2008).

Apart from rapid efflux of drugs mediated by cellular detoxification mechanisms, elevation in expression of factors involved in repair of damaged DNA also confers chemoresistance in ovarian cancer.

***Enhanced DNA repair mechanisms***

DNA adducts formed in tumor cells on exposure to chemotherapeutic drugs activates various DNA repair mechanisms, including nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), non-homologous end-joining (NHEJ) and homologous end-joining (HR) pathways (Ling, 2005; Martin *et al.*, 2008). Enhanced rate of DNA repair results in chemotherapy failure.

NER pathway predominantly repairs cisplatincarboplatin invoked intrastrand and interstrand DNA adducts. DNA adduct is usually formed in a single strand that is recognized by excision repair cross complementation (ERCC1) protein. After removing the lesion, DNA polymerase uses undamaged single strand as a template to resynthesize complementary sequence and the ligase seals the nick to complete repair of DNA. Selvakumaran *et al.* (2003) demonstrated that NER facilitates cisplatin induced DNA damage in ovarian cancer cell lines A2780, OVCAR-4 and OVCAR10. Resistant cell lines of OVCAR10 and OVCAR-4 showed higher expression of ERCC1, and antisense RNA against ERCC1 converted cisplatin-resistant OVCAR10 cells to cisplatin-sensitive. The study demonstrated that cytotoxic action of cisplatin may be enhanced by altering expression of factors involved in NER pathway (Selvakumaran *et al.*, 2003).

Mismatch repair (MMR) pathway removes mismatched bases incorporated through insertion and deletion by DNA polymerase, and has often escaped proof-reading mechanisms. Three steps involved in MMR are initiation, excision and resynthesis that are regulated by several Mut proteins, viz., hMSH1, MLH1, MSH3, MSH6 and PMS2.

Effective removal of tumor cells is dependent on active MMR pathway. However, methylation of *hmlh1* gene resulting in inactivation of MMR, causes resistance to platinum drugs and consequent poor survival (Ling, 2005; Martin *et al.*, 2008; Richardson *et al.*, 2005). BER pathway removes non-bulky damaged DNA bases, abasic sites and DNA single strand breaks (SSBs) that occur on exposure to alkylating drugs and other chemotherapeutic drugs (Kinsella, 2009). Fishel *et al*. (2007) reported that combination of temozolomide and methoxyamine (BER pathway inhibitors) invoked higher cell death in ovarian cancer cell lines IGROV-1, OVCAR-3 and SKOV-3 (Fishel *et al.*, 2007). The study emphasized that chemotherapeutic drugs in combination with inhibitors of BER pathway may potentiate ovarian cancer treatment.

Numerous factors such as ionizing radiation, reactive oxygen species and genotoxic chemicals cause SSBs, which when left unrepaired may form double strand breaks (DSB) in the S-phase of the cell cycle, causing cell death. Homologous repair (HR) and NHEJ pathways ensure repairing DSB and prevents cells from dying. DSB repair pathways are mediated by numerous genes including: *brca1*, *brca2, atm, atr, rad50, mre11, nsb1* and *fanc.* Mutation in *brca1* and *brca2* has a 15–40% increased chance of being afflicted with ovarian cancer. Expression of BRCA1 and BRCA2 varies in histological subtypes of ovarian cancer as well (Cerbinskaite *et al.*, 2012). A study analyzed DNA repair related genes: *parp1, ercc1, xpa, xpf, xpg, brca1, fanca, fancc, fancd2,* and *fancf* in 77 stage I, 88 stage III and 13 borderline ovarian carcinomas by real-time analysis. Expression levels of ERCC1, XPA, XPF and XPG were higher in stage I than stage III samples, thus correlating with advanced stage of disease. Whereas, BRCA1, FANCA, FANCC, FANCD2, and FANCF were lower in borderline and stage I than stage III samples. Also, patients with highest level of ERCC1 and BRCA1 when treated with platinum based therapy demonstrated better progression free survival than those treated with a combination of platinum and taxol, thus, indicating a role for DNA repair genes in overall and progression free survival in ovarian cancer patients (Ganzinelli *et al.*, 2011). Although numerous studies are being conducted to decipher factors that contribute to chemoresistance, the need of the hour is to establish personalized chemotherapy regimes.

**Chemoresponse assays**

Several exploratory research projects have been undertaken to establish chemoresponse assays to predict PFS and OS, and measure sensitivity to particular chemotherapeutic drugs to limit unnecessary expenditure, and aid in establishing personalized treatment regimen (Rutherford *et al.*, 2013). Numerous chemo-response assays such as differential staining cytotoxicity assay (DiSC), extreme drug resistance assay (EDR), histoculture drug resistance assay (HDRA) and adenosine triphosphate (ATP) bioluminescence assay have been developed that share four common steps: (1) isolation of cells from tissue, *in vitro*

on medium or soft agar; (2) incubation of cells with several drugs at different concentrations; (3) inspection of cell survival fractions; and (4) analysis of obtained results.

A recent study used ChemoFX assay in a non-interventional, unbiased clinical trial on 262 ovarian cancer patients. The tumor samples were collected at time of recurrence and sent for *in vitro* analysis and simultaneously treatment regimens were initiated. Fifty five percent patients bore platinum-sensitive recurrent EOC where high grade papillary serous tumors were most abundant. Both single and dual agent combination chemotherapies to a maximum of four cycles were administered and 25-30% patients responded to the treatment. More than 50% of tumors were found to be responsive to minimum one drug tested *in vitro*, indicating that chemoresponse assay based informed personalized chemotherapy may benefit platinum-sensitive and platinum-resistant recurrent EOC patients (Rutherford *et al.*, 2013).

**Molecular imaging modalities**

Apart from using biomarkers and laproscopy, analysis of IP infiltration, non-invasive molecular imaging technologies like CT, magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), diffusion weighted imaging (DWI) are routinely used to determine the stages in ovarian cancer. Recently, a comparative study on imaging techniques (Doppler ultrasonography CT, PET/CT, MRI) on 132 ovarian cancer patients identified 95 malignant tumors, 13 borderline tumors and 25 benign tumors. The study highlighted PET/CT as a preferred technique as it showed higher sensitivity (91.6%), specificity (81.6%), PPV (92.6%), and NPV (79.5%) in detecting malignant tumors. Precision of PET/CT in detecting benign cases versus those that are borderline/malignant was higher than Doppler ultrasonography, MRI or CT (Nam *et al.*, 2010). Apart from using PET/CT combination, PET alone showed immense diagnostic potential to detect tumors in patients with indecisive transvaginal ultrasonography, presence of metastases and aid in staging of ovarian cancer (Musto *et al.*, 2011). Other than $^{18}$F-FDG, molecules like 16-[18F] fluoro-17-estradiol (FES), $^{11}$C-Choline, and $^{15}$O-PET are actively used to assess ovarian tumors (Tsujikawa *et al.*, 2008).

Recent progress in imaging modalities is demonstrated by a novel method called optical coherence tomography (OCT) utilizing near-infrared as source of light for non-invasive diagnosis. Hariri and colleagues were the first to combine OCT with routine laproscopy (LOCT) to differentiate normal ovary, epithelial ovarian carcinoma, and endometriosis. Further, combination of OCT with ultrasound guided transvaginal imaging may pave way for less invasive methods to visualize uterine endometriosis (Hariri *et al.*, 2009).

Due to limitations with anatomic imaging through CT and MRI scans in identifying tumors, functional imaging is gaining prominence in gynecologic cancers

(Motoshima *et al.*, 2011). DWI is a non-invasive functional MRI method (DWMRI) that determines diffusion of water molecules in tumors, providing information on density, volume and size. Differences in cellularity of tumors also enables differentiating benign and malignant tumors (Motoshima *et al.*, 2011). DWMRI has immense potential in predicting cytoreductive success in patients diagnosed with advanced ovarian cancer with a sensitivity of 91.1%. DWMRI can facilitate visualization of solid tumors and malignant deposits by providing an increased contrast versus noise ratio (Espada *et al.*, 2013). Thus, DWI opens up new avenues to determine response of ovarian cancer patients to proposed treatments in real time.

**Novel chemotherapeutic drugs**

A major problem faced by ovarian cancer patients on successive exposure to platinum and taxol compounds is recurrence of tumor. In order to alleviate OS and PFS, various new drugs have been initiated in clinical trials. Epothilones, the metabolites produced by myxobacterium (*Sorangium cellulosum)* is under investigation in various clinical trials for cytotoxicity in cancer cells. Six types of water soluble epothilones (A to F) inhibit microtubule function by preventing depolymerization of microtubules, initiating cell cycle arrest at G2/M phase, similar in action to paclitaxel (Reichenbach *et al.*, 2008). Currently five epothilones (ZKEPO, ixabepilone, patupilone, KOS-862 and BMS-310705) are in clinical trials.

Trabectedin or Yondelis extracted from *Ecteinascidia turbinate* (a marine sea squirt) induces apoptosis by producing superoxides which cleave DNA strand and invoke cell cycle arrest. A combination therapy on 337 platinum-resistant ovarian cancer patients showed 6–12 months of platinum-free hiatus compared to 335 patients treated with only PEGylated liposomal doxorubicin (PLD) (Krasner *et al.*, 2012). Krasner *et al*. (2007) also conducted a study on response rate to trabectedin in platinum-sensitive or platinum-resistant recurrent ovarian cancer patients. Patients were subjected to weekly infusion of trabectedin for 3 hours for three consecutive weeks followed by a week of no treatment. Sixty two platinum-sensitive patients showed a PFS of 5 months versus 2 months PFS in 79 platinum-resistant cases, while overall response rate (ORR) was 29% and 6.9% in platinum-sensitive and platinum-resistant patients, respectively.

Canfosfamide also called as *telcyta TLK286* was evaluated in combination with PLD in 125 platinum-resistant ovarian cancer patients in a trial (NCT00350948). PFS of 5.6 months and 3.7 months were achieved in combination treatment and only PLD treatment, respectively. Moreover, there was a lower incidence of palmar-plantar-erythrodysesthesia in patients subjected to canfosfamide + PLD than PLD alone (23% versus 39%) (Vergote *et al.*, 2010). A phase III clinical trial on 247 platinum-resistant ovarian cancer patients evaluated efficacy of a combination of canfosfamide + carboplatin against liposomal doxorubicin. The authors reported an overall response rate (ORR) of

31.6% versus 10% in canfosfamide + carboplatin against liposomal doxorubicin treatment, respectively (Rose, 2007).

**Targeted therapy for ovarian cancer**

In contrast to breast cancer, targeted therapy is still not a standard practice of care for ovarian malignancy. Bevacizumab (Avastin) is an antiangiogenic humanized recombinant monoclonal antibody that inactivates VEGF and is thought to prevent VEGF-mediated cell growth in tumors. Efficacy of bevacizumab was tested in Gynecologic Oncology Group (GOG) protocol 218 (GOG 218), a phase III placebo-controlled clinical trial in a cohort of untreated 1873 advanced stage epithelial ovarian cancer, primary peritoneal and fallopian tube cancer patients. The study reported a median PFS of 14.1 months in patients who received concurrent and maintenance bevacizumab along with carboplatin + paclitaxel against 10.3 months in patients treated with carboplatin + paclitaxel. A multi-centric phase III clinical trial, ICON-7 (International Cooperative Group for Ovarian Neoplasia) studied effect of bevacizumab in 1528 stage IAIIA and stage IIBstage IV ovarian cancer patients. Patients on bevacizumab along with carboplatin + paclitaxel showed 19 months median PFS versus 17.3 months in control group. Bevacizumab efficacy was examined in 484 patients with recurrent ovarian cancer in a phase III clinical trial called OCEANS. Patients treated with 6–10 cycles of bevacizumab + carboplatin + gemcitabine and carboplatin + gemcitabine + placebo showed a median PFS of 12.4 months and 8.4 months, respectively. However, adverse effects such as hypertension, gastrointestinal perforation caused due to use of bevacizumab, were observed in patients in all clinical trials. Besides, incorporation of bevacizumab along with other chemotherapeutic drugs did not improve OS of women diagnosed with ovarian cancer. Thus, US FDA did not approve the use of bevacizumab as standard practice in the treatment of ovarian cancer (Eskander *et al.*, 2013).

Pazopanib (Votrient) prevents angiogenesis by inhibiting VEGF receptors (VEGFR1, VEGFR2, and VEGFR3), platelet-derived growth factor receptor (PDGFR), and C-Kit. Phase II clinical trial, is currently underway to measure efficacy of pazopanib in combination with topotecan on patients presenting with recurrent epithelial ovarian cancer, fallopian tube cancer and peritoneal cancer (NCT01600573). A drug called Olaparib (AZD2281) binds to poly (ADP-ribose) polymerase (PARP) and inhibits DNA repair mediated by PARP. A phase III clinical trial (NCT01844986) is underway to understand efficacy of Olaparib in ovarian cancer patients carrying *brca* mutation and treated with platinum-based chemotherapy.

Failure of chemotherapy with first line of platinum drugs has prompted investigations on establishing chemosensitive and chemoresistance assays to determine response of ovarian tumor to second-line chemotherapeutic drugs (Jordan *et al.*, 2013). Progress in chemoresponse assays will herald an era of personalized regimen of

chemotherapy that may benefit ovarian cancer patients. It is anticipated that translation of potential drugs from bench-to- bedside will not only improve OS rate and progression free survival but will also extend the current five-year survival rate.

**Future directions**

The 21st century has witnessed significant advances in diagnosis, therapy and disease management in ovarian cancer that has reduced the overall mortality rate. Ovarian cancer is not an exception as five-year survival rate has increased over the last 30 years, however, the final solution is still not in sight. The survival rate varies greatly according to how early the disease is diagnosed. Extensive research on identifying new tumor or serum-based biomarkers is in progress worldwide, and several promising candidates like HE4 are either in clinic or ready to enter the clinical trials. It is now obvious that not one but a combination of biomarkers will probably be the future choice after extensive validation in large cohorts, with advanced technologies and well designed assays.

Although early stage ovarian cancer patients have the potential to live a disease free life, women with advanced disease and recurrent disease require better treatment. Advanced imaging techniques combined with targeted therapy to tackle the tumor burden for optimal debulking surgery seems a thrust area. Many newer imaging modalities such as DWMRI, LOCT along with the standard PET/CT are being adapted in clinic. A quest for therapeutic molecules to target advance and subtype specific ovarian cancer is ongoing. For the relapse cases, the need is again on developing alternate therapeutic molecules based on detailed understanding of drug resistance of the cells. A focus on early detection of acquired chemoresistance needs to be actively pursued to alleviate the cytotoxic effects of platinum–taxol therapy. High-throughput genomic analyses, phage or antibody display techniques may add in identifying markers to detect patient population acquiring resistance towards the standard therapy. Globally, increased focus on various pathways to ovarian cancer and modalities towards early detection, better prognosis and management of the cancer patients is anticipated. The hope is to shift the paradigm for ovarian cancer from a more controlled chronic disease to an ultimate cure.

**CONFLICT OF INTEREST**

The authors claim no conflict of interest.

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# Single Nucleotide Polymorphisms in Human Health and Disease: Towards Resolution of a Conundrum

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Single nucleotide polymorphisms, also referred to as Single nucleotide variants remain single most dominant feature of variations in the human genome. The large numbers, stability and presence at easily detectable frequencies make them the most suitable potential markers for association studies in human health and disease. The study of these common genetic variants has now evolved and given rise to the understanding of less frequently encountered 'rare' variants of varying minor allele frequencies. The acceptance of role of rare single nucleotide variants in human diseases appears to coincide with the phasing out of the term 'mutation' in the dbSNP. The contribution of rare variants helps resolve the enigma of heritability of complex traits to a great extent. Integration of analysis of rare and common variants with the support of technological innovations and improved strategies for data analyses is expected to yield better and reliable association data. By virtue of their relatively stronger impact on the phenotype compared to that of the common variants, rare variants are likely to be better candidates as markers of association as well as targets for intervention strategies while providing mechanistic insights into the underlying biological processes. Starting with discussion of the basics of single nucleotide variants, this review summarizes concepts and principles of approaches used to study their association with parameters in health and diseases including cancer. Implications for studies in Indian population are discussed.

## INTRODUCTION

Variations in individual members of a species and their inheritable characteristics has been recognized and documented for over centuries. However, the basis and mechanisms of transmission of such traits via genetic elements have been elucidated in the last few decades. This has primarily been achieved for traits that are associated with single genes. The identification of genetic elements associated with a wide spectrum of multigenic disorders or complex traits remains to be deciphered. Rapid advances in biotechnology and bioinformatics have now brought them into realm of reachable targets.

Of the various types of common genetic variations, namely, the single nucleotide polymorphisms (SNPs, called 'snips'), insertion-deletions (INDELs), and copy number variations (CNVs), SNPs emerge as the best option for clinical and research applications due to simplicity of stable (compared to other mutable tandem repeat polymorphisms) biallelic patterns. Moreover, the alleles are often present in population at frequencies in excess of 5% and can be easily determined by PCR with small amplicons (Brookes, 1999). The availability of an

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extremely large array of variants that can be analyzed economically in multiplexed formats further enhances the utility of SNPs. The analysis of human genome published in 2001 reported identification of 1.42 million variants (Sachidanandam *et al*., 2001), which has escalated to 15 million recently (Genomes Project Consortium *et al*., 2010) and the latest dbSNP release documents identification of over 60 million uniquely identified SNPs (NCBI dbSNP Build 138, 2013). The increasing numbers further support the use of SNPs in disease association studies. Such analyses can help understand the basis for the phenotype and support development of tests to identify high risk individuals and aid in prediction of prognosis in affected individuals in case of common disorders. In addition the data could provide clues to identify targets for the development of intervention strategies.

**What are SNPs?**

A single base pair mutation at a particular site in the genome that is present in > 1% of the population is referred to as a SNP. These are primarily biallelic with exception of a few triallelic SNPs (Hodgkinson and Eyre-Walker, 2010). The number of SNPs across human genome is so large that they account for over 80% of the difference between any two individuals, which enhances their utility as most informative targets to examine association with variations in health and diseases. Individual SNPs are designated by a *reference SNP* number (*rs*) that represents a unique identity. At molecular level, SNPs are of two types; *Transitions* which involve replacement of pyrimidine base by pyrimidine base (or purine by purine), and *Transversions* that involve replacement of a pyrimidine base by a purine base or vice versa. Transitions (A/G or T/C) are the most commonly encountered substitutions (Brookes, 1999).

With regards to locations, the SNPs can occur in coding or non-coding regions and their impact on the gene expression or protein function, in other words phenotype, would depend upon their location and whether they involve synonymous or non-synonymous codon changes. While the synonymous SNPs have been perceived as benign, a considerable body of data shows that such changes in non-coding regions involving sites participating in gene regulation or mRNA processing may impact the genome, mRNA transcripts and functional/structural proteins (Chamary *et al*., 2006). SNPs in noncoding regions, can also serve as important genetic or physical markers for comparative or evolutionary genomics studies. SNPs, when present in regulatory sites of a gene, can affect rates of transcription causing changes in the levels of the expressed protein. In the coding regions, SNPs can affect protein structure and hence function, resulting in an altered response to a drug or environmental toxin. Such SNPs can serve as molecular markers in several diseases and pharmacogenomic studies.

**SNPs: Where do they come from and where do they go?**

It is estimated that substitution mutations may occur at the rate of ~$10^{-8}$ per base pair per generation. SNPs thus can arise, *de novo* with

transition substitution being the most common type of variation (particularly C/T) (Genomes Project Consortium *et al*., 2010). The incidence of SNPs has also been found to vary across the genomes with GC rich regions showing 15% higher heterozygosity compared to those with low GC content. X and Y chromosomes were found to show lower frequency of SNPs compared to autosomes. The lower mutation rate in females and smaller effective population size (as compared to autosomes) may account for the lower rates in X and Y chromosomes. It has been estimated that following introduction in the genome, a possibility of over 50% exists that such mutations are lost in the next ten generations or approximately two hundred years (Miller and Kwok, 2001). A comparative analysis of human and Orangutan X-chromosomes that showed no shared SNPs, led to the inference that life time of SNPs is shorter than the time taken for divergence of the species (over 10 million years). An average of 284,000 years was estimated as lifetime of a SNP by Miller *et al.* after taking into account the divergence and variability amongst humans (Miller *et al*., 2001).

The principles of natural selection are considered to favor prevalence of alleles that improve health of subjects and eliminate lethal or harmful alleles. However, deleterious alleles appear to survive and spread, a phenomenon that can be explained on the basis of three mechanisms primarily proposed for survival of a new SNP (Hurst, 2009). First, since DNA segments are inherited as blocks, the resulting linkage disequilibrium (LD) may not allow the alleles to segregate independently. This non-independence leads to background selection wherein loss of linked allele also results in the loss of deleterious alleles or vice-versa termed as *'hitchhiking'* when selection of deleterious allele occurs in association with a positively selected allele. The Non-Mendelian inheritance patterns involving favored transmission of an allele to more than 50% of the progeny or uniparental transmission of cytoplasmic factors, constitutes the second mechanism. Finally, a complex phenomenon of *kin selection* proposes that deleterious alleles can survive if they promote fitness of other members of the community (Hurst, 2009). Thus, a substitution would have a greater chance of survival if it is in LD with a conserved, functionally critical allele or it becomes homozygous in some individuals.

The selection mechanisms can also be envisaged in the context of their influence on resultant allele frequencies (outcome). Positive selection or directional selection leads to spread and fixation of a beneficial allele in a given population or a species. On the other hand, negative or purifying selection leads to elimination of a deleterious or weaker allele. Finally, a heterozygosity derived survival advantage would lead to maintenance of both the alleles in the population and this type of selection is termed as balancing selection.

The ratio of non-synonymous changes in a segment of gene of interest to synonymous changes in the flanking or other areas of the genome (NS:S) is used to determine if the

particular locus is under positive or purifying selection. This is based on the premise that non-synonymous changes that do not affect protein function would be fixed at the same rate as synonymous changes (NS:S = 1). Thus, for SNPs in a given set of genes or a DNA segment, a ratio of NS:S > 1 would favor the possibility of positive selection; whereas NS:S < 1 favors the possibility of purifying selection (Massingham and Goldman, 2005). The genes harboring the latter class of variants under purifying selection include deleterious variants relevant for health and disease associations. Incidentally, relatively higher frequency of non-synonymous substitutions has also been interpreted as a basis of adaptive evolution (Bielawski *et al*., 2000). Thus, selection of an allele can lead to a lower or higher frequency of individuals homozygous for that allele. Tests such as Ewens-Watterson homozygosity test of neutrality, using haplotype data under the assumption of Hardy Weinberg Equilibrium (HWE), allows discrimination between balancing (observed homozygosity < expected) or directional (observed homozygosity > expected) selection (Kaur *et al*., 2007). However, one must interpret results of such analyses in the context of possible population bottleneck, subdivisions, genetic drift and non-random mating (endogamy), as these also lead to excess of homozygosity obscuring the underlying selection processes.

**Rationale for the use of SNPs as markers in disease association studies**

Familial occurrence of several autoimmune as well as other common disorders such as schizophrenia or obesity is a common observation, although these do not display typical strong Mendelian pattern of inheritance ruling out contribution of one or few genetic elements to the predisposition. Based on the initial results of the human genome project and lower sequence variations in coding regions, it was proposed that genetic factors represented by specific alleles contributing to the risk for common ailments may be common in the population (Lander, 1996). This argument formed the basis of '*common disease, common variants*' hypothesis. It was also demonstrated that association studies and not linkage analyses (family based) will be better suited for examining the link between diseases and common variants, as these contribute to a small increase in the relative risk (≤ 20%). The analysis also revealed that large numbers of SNPs and large cohort sizes should be a prerequisite for such studies to achieve adequate statistical power and hence the concept of halpotype based association analyses was proposed (Risch and Merikangas, 1996).

**Polygenic model and identification of high-risk subjects**

The considerations relevant to polygenic traits apply equally well to malignant disorders which often show heritable pattern with higher incidence in first degree relatives. The germline mutations in various cancer susceptibility genes not only can account < 10% of all cancers (Rahman and Stratton,

1998), but also only half of the familial cases, especially where a number of moderate to low penetrance gene mutations have been implicated, e.g. breast cancers (Cox *et al*., 2007; Turnbull *et al*., 2010). Thus, the genetic basis for a significant proportion of familial cases remains poorly understood. The cancers arising in patients with familial history display several features with those arising in subjects without any history of cancer in first degree relatives. Therefore, the knowledge of the genetic basis in the former category of patients could help identify high risk subjects in general population that constitute 90% of the sporadic cases. The evidence from studies in cancer families as well as from animal models have highlighted the importance of modifier functions in determining penetrance of mutations in tumor suppressor genes (Kwong and Dove, 2009; Couch *et al*., 2013). The SNPs that result in low to moderate change in the expression or function of a protein, come across as ideal candidates as these enhance or reduce the influence of a deleterious mutation in a tumor suppressor gene that are characterized by pleiotropic effects.

Theoretical basis for the additive effect of common variants with low penetrance in determining cancer risk was provided by elegant analysis of data from population based and BRCA1/2 negative familial cancer studies by Antoniou *et al*. (2002) that strongly supported the polygenic nature of the disease. Further, extension of this polygenic model to examine distribution of risk in general population revealed that as small as 12% of the population at the highest risk accounted for nearly half of the cases (Pharoah *et al*., 2002). These considerations support the use of a panel of low-penetrance alleles for the identification of high-risk subjects which could make population based screening programs efficient and economical (Pharoah *et al*., 2008).

The understanding of the basis of emergence and existence of common variants and technological developments led to a surge in studies examining association between common genetic variants and a variety of diseases extending up to relatively uncommon Autism spectrum disorders (Klei *et al*., 2012). Typically, the frequency of these variants is expected to be > 5% with a very modest effect of 15–20% increase in the disease risk (OR ≤ 1.5) with ambiguous and often conflicting association data suggesting influence of a variety of other factors (Cox *et al*., 2007; Kaklamani *et al*., 2005). It was also realized that despite expected levels of associations, these variants could account only for small fraction of disease heritability (Manolio *et al*., 2009). Thus, it became clear that the number of alleles contributing to risk for common ailments could be much larger and each one of them may have variable individual contribution.

**Rise of rare variants**

Presence of low frequency variants became evident from studies which showed that the number of variants detected was a function of the sample size and thus larger studies permitted detection of higher number of variants (Nelson *et al*., 2012). Thus, the

explosion of studies of common variants for disease associations was accompanied by recognition of a large number of functional genetic variants present at much lower frequencies and their possible relevance for common diseases. As a general rule, SNPs with frequencies < 0.5% are referred to as rare variants (Nelson *et al*., 2012). More stringent analyses use minor allele frequencies (MAF) < 0.01% as a cut off to define rare variants; whereas those between 0.01% and 0.05% are considered low frequency with the remaining as common variants (Li and Leal, 2008). A recent report proposed that the MAF cut off for defining rare variants may be determined by the sample size such that the MAF for the rare variants is defined as equal to $1/\sqrt{2N}$ (Ionita-Laza *et al*., 2013).

The role of rare variants was in fact envisioned early in the past decade by Pritchard, with an approach of considering selection pressures and relatively low penetrance/effect of the susceptibility alleles. He postulated that, a) alleles contributing to a complex phenotype would be spread over multiple loci; b) their frequencies would vary randomly; c) the susceptibility imparting mutations will be mildly deleterious; and hence d) these may be subject to weak selection (Pritchard, 2001). A study by Kryukov et al. (2007) extended these arguments by defining three different categories of harmful mutations as, a) 'damaging' mutations that reduced protein function marginally; b) 'detrimental' mutations that would predispose to a disease; and c) 'deleterious' mutations that result in loss of function and are likely to be subjected to purifying selection. Due to weak effects on the protein function, the first two types of variants may not be under strong selective pressure/purifying selection and hence may persist in the population. Thus, presence of many different clinically relevant mildly detrimental variants at high combined frequency could underline the prevalence of many complex disorders (Kryukov et al., 2007). The analyses laid the foundation of the concept that the rare, non-synonymous substitutions are the best potential targets and that re-sequencing of candidate genes in adequate numbers of subjects could help identify these entities. Such disease associated SNPs would be characterized by frequencies in the range of 0.1–2%, with ORs > 2, may lack familial concentration and will require resequencing of the candidate gene segments as these will not necessarily be highlighted in Genome-wide association studies (GWAS) (Bodmer and Bonilla, 2008). A recent study of sequencing of 200 genes coding for proteins targeted by various drugs in over 10,000 subjects found that rare variants (SNVs) were abundant and their presence was characterized by concentration in geographically related population. The study also noted that these variants had strikingly lower frequency of non-synonymous substitutions (NS:S = 0.54) implying that the loci were subject to strong purifying selection justifying their selection as drug targets (Nelson et al., 2012).

The evidence for presence of clinically important rare variants has been slowly accumulating over a decade. The earliest being

those present in the genes associated with plasma levels of high-density cholesterol (Cohen et al., 2004) and hypertension (Ji et al., 2008). In this case a common SNP was identified in GWAS, whereas four rare variants were identified by sequencing studies in familial and other cases. More importantly, the linkage disequilibrium between these variants was low indicating that they exerted protective effect independent of each other. The importance of rare variants has also been highlighted by their contribution to risk for Type1 Diabetes (Nejentsev et al., 2009).

**Implications of common and rare variants associated with complex diseases**

Bodmer and Bonilla (2008) implicate common variants as markers of rare variants when in LD with functional rare variants. It is also possible that a functional common variant may influence the effect of a rare variant and thus act as its modifier. The influence of variety of modifiers on the penetrance of mutations in cancer susceptibility genes (rare variants) could represent the latter phenomena. Data in this regard indeed suggests marginal influence of common variants on the risk due to mutations in cancer susceptibility genes (Antoniou *et al*., 2008; Couch *et al*., 2013). In the context of determining cumulative risk, the authors argue that due to relatively higher penetrance, rare variants could be more important players compared to the common variants. Thus, the rare SNPs are the preferred candidates in screening tests for the identification of high risk subjects or for application of interventional modalities and replace the common variants (SNPs) as suggested by earlier studies (Pharoah *et al*., 2008). Gibson proposed three more possible scenarios that include contribution of rare variants (Gibson, 2011). For a disease with a rare allele based effect, it is proposed that the number of disease associated loci would depend upon the incidence of the disease, number of variants per locus, minor allele frequencies of individual variants and the effect size for each allele (ORs). Each allele is expected to have a relatively large effect and the risk rise with increasing numbers of alleles implying high disease heritability. According to another 'Infinitesimal Model', a large number of loci with very small effects may contribute to the disease risk. Herein, the heritability may be missed primarily because of the very low effect associated with each variant that is lower than the threshold used to identify risk alleles. Such a scenario is typically highlighted by observations describing identification of several additional loci associated with human height when sample size was increased from 30,000 to 180,000 subjects (Lango Allen *et al*., 2010). Finally, a situation may exist that represents, a 'broad sense heritability' model, wherein non-additive gene-gene, gene-environment interactions and epigenetic events may have a role in determining risk for the disease.

**Challenges in the application of SNP studies in Indian scenario**

It has been proposed that the genome of population in the Indian subcontinent arises

from the amalgamation of three major lineages, the Austro-Asiatic, Dravidian and Indo-Europeans (Reich, 2009). The first population based genome variation analysis highlighted high levels of differences across different ethnic groups with each group exhibiting considerable level of homogeneity when examined within geographically and linguistically similar populations (Indian Genome Variation Consortium, 2008). A subsequent genome wide analysis of over hundred subjects from different regions of India yielded data that implies presence of two major lineages in which the component close to west European has been defined as Ancestral North Indian (ANI); while the other component dominant in Dravidian population is referred to as Ancestral South Indian (ASI) with indigenous Andamanese (Onge) as its nearest genetic relatives. More importantly, the presence of these two components was seen in all the subjects irrespective of ethnicity, cast or geographic location (Reich, 2009). Further, the ratio of the two ancestral components was dictated primarily by geographical location followed by caste structures, with contribution of ANI component varying between 71% and 31% in a North-South gradient. The results also provided evidence for genetically diverse subgroups arising through relatively recent founder events where the founder signature has been maintained presumably due to endogamous behavior.

These interpretations are perfectly compatible with the generally known features of the population in the Indian subcontinent. It implies the need for additional care in study design and strict matching between cases and controls for the study of common as well as rare variants. Several studies have highlighted the significance of matching between the study groups with careful documentation of geographical and ancestral origin of the subjects (Nelson *et al*., 2012; Do *et al*., 2012). Nelson and colleagues detected one rare variant every 17 bases which were highly geographically restricted (Nelson *et al*., 2012). Based on the data from GWAS studies, global nature of the common variants is often highlighted, though the MAF of common variants can differ significantly across ethnicities (Swallow, 2003; Shriner, 2009; Van Dyke *et al*., 2009; Waters *et al*., 2010a; Waters *et al*., 2010b; Gibson, 2011). A common and an extensively studied SNP *rs1800795* located in the promoter region of the IL-6 gene, (IL-6 -174G/C) is absent or present at very low frequency in Eastern Asian populations (Zhai *et al*., 2001; Hayakawa *et al*., 2002). Hence, to ensure that the effects of genetic and environmental background are evenly distributed in the study groups, an extremely stringent cataloging of demographic features and selection as well as matching of subjects in the study groups is warranted. The functionality of most SNPs in Indian populations also remains to be delineated. This is particularly relevant for variations in the non-coding regions where adjacent SNPs may influence the gene expression in a way that may not be easily understood (Smith and Humphries, 2009).

**SNP studies in cancer in Indian populations**

A report over two decades ago by Saranath *et al.* (1990) identifying an allele associated with the risk of aggressive disease in oral cancer patients represents the first major study of gene polymorphism in cancer patients from India. Since then to date, more than five hundred peer reviewed articles (Pubmed) have been published that deal with diverse array of genes belonging to different classes such as, tumor suppressor genes (Hedau *et al*., 2004; Kannan *et al*., 2000; Pramanik *et al*., 2011; Ayub *et al*., 2014; Juwle and Saranath, 2012; Kotnis *et al*., 2012), xenobiotic metabolism (Anantharaman *et al*., 2007; Chacko *et al*., 2005; Joseph *et al*., 2006; Ihsan *et al*., 2011), DNA repair (Ramachandran *et al*., 2006; Kumar *et al*., 2012; Mahimkar *et al*., 2012; Mandal *et al*., 2010; Mondal *et al*., 2013), and inflammation (Upadhyay *et al*., 2008; Gangwar *et al*., 2009; Gaur *et al*., 2011a; Gaur *et al*., 2011b; Singh *et al*., 2012; Joshi *et al*., 2011; Joshi *et al*., 2013a). Oral cancers with a single GWAS by Saranath and coworkers (Batnagar *et al*., 2012) remain the most frequently studied category, followed by breast cancers. Of significance are reports that document presence of novel SNPs in IL-6 (Saha *et al*., 2003), K-RAS (Pramanik *et al*., 2011), and RET (Sharma and Saranath, 2011) genes. While the available data thus supports the influence of polymorphisms in genes associated with various pathways on the risk for malignant disorders in Indian populations, the novel SNPs could represent ethnicity specific common polymorphisms or rare SNVs characteristic of the population studied.

A trend emerging out of this large body of data strongly implicates inflammation associated gene polymorphisms as potential risk modifiers with their magnitude of influence especially significant and relevant for Indian population. These association studies in the context of risk for various malignancies (Upadhyay *et al*., 2008; Gangwar *et al*., 2009; Gaur *et al*., 2011a; Gaur *et al*., 2011b; Singh *et al*., 2012; Joshi *et al*., 2011; Joshi *et al*., 2013a) imply that the diverse range of variants that influence pro- or anti-inflammatory responses may represent a common denominator as risk modifiers for cancers. Similar influence for autoimmune and infectious diseases may also be present (Aggarwal *et al*., 2011; Ahluwalia *et al*., 2009). A majority of the data remains predominantly derived from studies in populations with dominant ANI genetic makeup as exemplified by studies in relation to one of the most widely studied SNPs located in the promoter region of IL-6 (Personal communication). The observed trends and their proposed influence are supported by several lines of evidences relating to selection of genotypes by environmental pressure, burden of infection (Le Souef *et al*., 2000; Blackwell *et al*., 2005; de Martel *et al*., 2012) as well as ethnicity associated variations in a variety of parameters (Chandalia *et al*., 2003; Battle *et al*., 2007; Spielman *et al*., 2007).

Indian population structure shaped by castes and endogamy over the past few centuries also raises exciting possibilities in the context of rare variants as these represent recent events and are highly restricted in terms

of population distributions. Community specific rare variants could serve as markers for certain biological associations and thus aid personalized genome based modalities. The genome diversity also promises myriads of opportunities to study gene-gene or gene-environment interactions.

## SUMMARY

The identification and acceptance of the rare variants as influential entities in human health and disease has resolved the confusion associated with limitations in the use of common genetic variants. The emerging information and the concepts derived thereof also add to the awareness of the complexities of the system which has immensely helped in study designs, methodologies, data analysis and interpretation. With significantly enhanced understanding the association studies can now be viewed in the context of a variety of possible scenarios. A two tier approach seems to be involving where GWAS based studies could help identify common variants which may reside in coding or non-coding regions and help identify potential target genes or regions for detailed analysis; while the candidate-gene based approach with exome sequencing is the most promising way to identify variations in the relevant coding regions. These could then be assessed for biological basis by a variety of means including molecular analysis with cost effective affordable assays, as routinely used in biochemical analyses. The need for big data bases and high throughput technology are two important imperatives that make such analyses feasible which also emphasize a need for collaboration between the industry and academic institutions.

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## CONFLICT OF INTEREST

The authors claim no conflict of interest.

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# Modulation of Proliferation by Gonadotropin-Releasing Hormone Receptors in Breast Cancer Cells

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Gonadotropin-releasing hormone (GnRH) is secreted from hypothalamic neurons and bind to receptors on gonadotrope cells of the pituitary gland, which then synthesize and release luteinizing hormone and follicle-stimulating hormone that regulate gonadal development. The presence of GnRH receptors and the effects of synthetic analogs of GnRH at extrapituitary sites is less clear. Several reports suggest that GnRH/analogues through cognate receptors may regulate mitogenic responses in cancer cells in an autocrine or paracrine manner. However, the inherent intracellular signaling pathways triggered are unknown. Using a highly specific antibody to human GnRH receptor we show that T47D breast cancer cells express GnRH receptors on their surface and that a GnRH analogue Cetrorelix inhibits proliferation of these cells, possibly via inhibition of processes that trigger cAMP formation.

## INTRODUCTION

Gonadotropin-releasing hormone (GnRH), a decapeptide hormone is the key regulator of the reproductive processes in both sexes. The hormone is secreted by hypothalamic neurons in a pulsatile manner and activates the GnRH receptor, a G-protein coupled receptor (GPCR), on gonadotrope cells of the anterior pituitary. It stimulates the release of the gonadotropins, luteinizing hormone, and follicle-stimulating hormone (Clayton and Catt, 1982; Hazum and Conn, 1988). The gonadotropins together control the function of the gonads and thereby reproductive processes. GnRH and GnRH receptors are expressed at a number of extrapituitary sites as well. These include various parts of the brain (Hsueh and Schaeffer, 1985), placenta (Khodr and Siler-Khodr, 1980), breast (Butzow *et al*., 1987; Harris *et al*., 1991; Palmon *et al*., 1994; Sarda and Nair, 1981; Seppala and Wahlstrom, 1980), prostate (Azad *et al*., 1993b), gonads (Hsueh and Schaeffer, 1985), activated lymphocytes (Azad *et al*., 1993a) and tumors of the reproductive system (Limonta *et al*., 1993; Miller *et al*., 1985; Moody *et al*., 1998; Ohno *et al*., 1993). GnRH analogues have been widely used in the clinical management of human malignant tumors of the ovary, endometrium, breast and prostate (Eidne and Anderson, 1996; Csernus *et al*., 1999; Schally, 1999). The rationale for this is that chronic administration of potent GnRH agonists and antagonists leads to decreased gonadotropin release from the pituitary, thus bringing about a general suppression of the pituitary-gonadal axis, leading to decreased steroidogenesis. As steroids support their growth, cell

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proliferation in tumors is affected. However, there is increasing evidence that GnRH analogues may have a direct anti-proliferative effect on cancerous cells via GnRH receptors expressed on them (Emons *et al*. 1997a). This and the finding that peripheral tissues locally produce GnRH/GnRH-like peptide (Emons *et al*. 1997b) and express GnRH binding sites prompted the idea that GnRH may exhibit an autocrine or paracrine role at sites distal to the pituitary. The exact role of locally secreted GnRH, GnRH receptors expressed in the context of specific tissues and intracellular signaling events is varied and not known in most cases. Since the GnRH receptors present on cancer cells were demonstrated to be identical in sequence to that in the pituitary (Kakar *et al*., 1994), it seemed reasonable that GnRH-mediated signaling pathways in extrapituitary tissues may be similar to the pituitary gonadotrope cells (Stojilkovic and Catt, 1995). Hence, studies were initiated in our laboratory to delineate effects of GnRH and its analogues on cancer cells. In earlier studies, we had used a receptor-specific monoclonal antibody (mAb) F1G4 to a synthetic peptide corresponding to amino acids l–29 of the human GnRH receptor to show that a subset of T47D cells express GnRH receptors (Karande *et al*., 1995). We confirmed the presence of GnRH receptor mRNA as well as expression of the receptors at the cell surface, measured the effects on cell division and studied the impact of GnRH analogues on intracellular cAMP and activation of growth factor responsive promoters in the breast carcinoma cell line T47D cells.

## MATERIALS AND METHODS

### Reagents

GnRH, [Trp$^6$] GnRH, Cetrorelix, Triptorelin, Buserelin, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trizol reagent (reagents from Sigma, USA). Epidermal growth factor (EGF), pertussis toxin, opti-MEM, lipofectin (reagents from Life Technologies, USA). Rabbit anti-mouse IgG-conjugated to fluorescein isothiocyanate (FITC) or horse radish peroxidase (HRP) (reagents from Dakopatts, Denmark ).

### Cell lines

T47D (human breast carcinoma cell line) and Cos-7 cells (monkey kidney cell line) procured from Vrije University, Amsterdam, and αT3-1 (mouse gonadotrope cell line) a gift (Mellon *et al*., 1990), were cultured in DMEM supplemented with 10% FBS, at 37 °C in a water-jacketed $CO_2$ incubator and passaged twice a week.

### RNA extraction and RT-PCR

Total RNA was extracted from cells using Trizol reagent and cDNA was synthesized using reverse transcriptase (Invitrogen Life Technologies, USA). PCR was carried out using a set of primers spanning all three exons of the hGnRH receptor (Figure 1a). The sense primer 5′ CAAAG TCGGA CAGTC CATGG 3′ and the antisense primer 5′ ATGAA GTGGC AAATG CAACC 3′ were used for amplification. Nested PCR was used with internal primers (sense primer: 5′ TGCAG

GACCA CAGTT ATACA TCTT 3′ and antisense primer: 5′ ATTCA GTTGT AGTTC GTGGG GGT 3′). Thirty-five cycles of PCR were carried out with denaturation at 94 °C, 1 min, annealing at 58 °C, 1 min, and polymerization at 72 °C, 1 min in a thermal cycler (Applied Biosystems, USA).

**Western blotting**

Cells were homogenized in 10 mM Tris-HCl, pH 7.2, containing 1 mM EDTA, 150 mM NaCl, protease inhibitor cocktail, and cleared by centrifuging at 300 × g for 10 min at 4 °C. The supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The crude membrane preparation in the pellet was electrophoresed on a 10% SDS-polyacrylamide gel under reducing conditions and proteins were transferred to PVDF membrane. The blots were incubated with mAbs followed by the secondary antibody conjugated to HRP. The blots were developed using enhanced chemi-luminescence (Millipore) and the images acquired using LAS3000 (GE Healthcare, USA).

**Transient transfection of Cos-7 cells**

Cos-7 cells were plated 80,000 cells per well in 24-well plates in DMEM containing 10% FBS, incubated overnight and cells washed with PBS. Five hundred nanograms of the plasmid, pcDNA1/Amp-GnRH R (GnRH receptor expression construct) and 2 µl of Lipofectin reagent were incubated in 200 µl of opti-MEM at room temperature for 45 min and then transferred to the cells. After 4 h, the medium was changed to 10% FBS-DMEM. After incubation for another 30 h, cells were trypsinized and cultured (5000 cells/well) for 24 h on Teflon coated slides.

**Immunofluorescence microscopy**

Cells were cultured on slides and fixed in methanol at -20 °C. They were incubated with the anti-GnRH receptor specific mAb F1G4, or the isotype control mAb, A9E4 (Karande *et al*., 1995), followed by incubation with the secondary antibody-FITC conjugate. The cells were mounted in 50% glycerol and immunofluorescent images captured using a confocal laser scanning microscope.

**Cell proliferation assay**

Cells were plated in 96-well plates at 7500 cells per well in DMEM containing 10% FBS. After 24 h incubation, the medium was changed to serum-free DMEM and treated with daily doses of the GnRH analogues, cetrorelix or buserelin for two days. This was followed by incubation with [$^{3}$H]thymidine ($0.2 \times 10^{6}$ cpm) for 12 h, cells were harvested onto glass fibre filters and incorporated radioactivity measured in a scintillation counter.

**Luciferase assay**

pfL711 is a plasmid containing the full-length 5′ regulatory sequences of the human *c-fos* gene (-711 to +39) upstream of firefly luciferase gene (Beckers *et al*., 1997). pRL-TK, a plasmid containing the herpes simplex virus thymidine kinase (HSV-TK) promoter upstream of Renilla Luciferase (Promega DLR™ Assay system, USA) was used as an

internal control. Cells ($8 \times 10^4$) were cultured in 24-well culture plates. The procedure for transfection of cells with pfL711 (500 ng) and pRL-TK (25 ng) was the same as outlined under 'transient transfection of Cos-7 cells'. The medium was changed 24 h later to serum free medium. After serum starvation for 72 h (the time required for endogenous activity of *c-fos* promoter to drop to minimal levels) in phenol-red free medium, the cells were treated with 100 nM EGF for 10 min. Luciferase activity was measured according to the protocol for the Dual luciferase™ assay kit (Promega, USA). Briefly, cells were lysed 6 h post stimulation with EGF by adding 100 µl lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol (DTT), 2 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100). After incubation for 15 min at RT, 20 µl cell lysate was added to 100 µl firefly luciferase substrate and the luminescence measured using a luminometer (Turner Designs, USA) in the dual mode. After this 100 µl Renilla Luciferase substrate was added and luminescence measured.

**cAMP radioimmunoassay**

Succinyl cAMP tyrosine methyl ester (ScAMP-TME) (Sigma, USA) was iodinated by the method of Steiner *et al.* (1972). For the immunoassay, 100 µl of appropriately diluted rabbit antibodies to cAMP (NIDDK-NIH, Bethesda, MD, USA) was incubated with varying concentrations of unlabelled cAMP (1–100 pmole) and $^{125}$I-labelled ScAMP-TME ($2 \times 10^4$ cpm) for 10 h at RT in a total volume of 300 µl of 50 mM sodium acetate buffer, pH 4.75. The antibody-bound radioactivity was separated from the free label by addition of 1 ml cold charcoal suspension 92 mg/ml in 100 mM potassium phosphate buffer, pH 6.3, containing 2.5 mg/ml BSA followed by centrifugation. The radioactivity in the supernatants was measured in a gamma counter (LKB model 1275, Minigamma, Sweden). Percent specific binding of the label was plotted against the concentration of the unlabelled hormones.

Cells were grown to 70% confluence in 24-well plates, washed with serum-free DMEM and 500 µM Isobutyl methyl xanthine (IBMX), a phosphodiesterase inhibitor was added. The cells were treated with GnRH analogues in serum-free medium for 30 min, cAMP was extracted from the cells with 0.1 M HCl and stored at -70 °C and measured by radioimmunoassay.

## RESULTS

### GnRH receptor mRNA expression in T47D cells

To detect GnRH receptor mRNA, reverse transcription and PCR were carried out using human GnRH receptor gene specific exon spanning primers (Figure 1a) (Wolfahrt *et al*., 1998) using total RNA from T47D cells. Although PCR using the first set of primers resulted in an expected product size of 368 bp, the band was not detectable on the agarose gel (Figure 1a). Therefore, a nested PCR was performed, and the 247 bpamplicon detected, suggesting that the GnRH receptor gene was transcribed in T47D cells (Figure 1a).

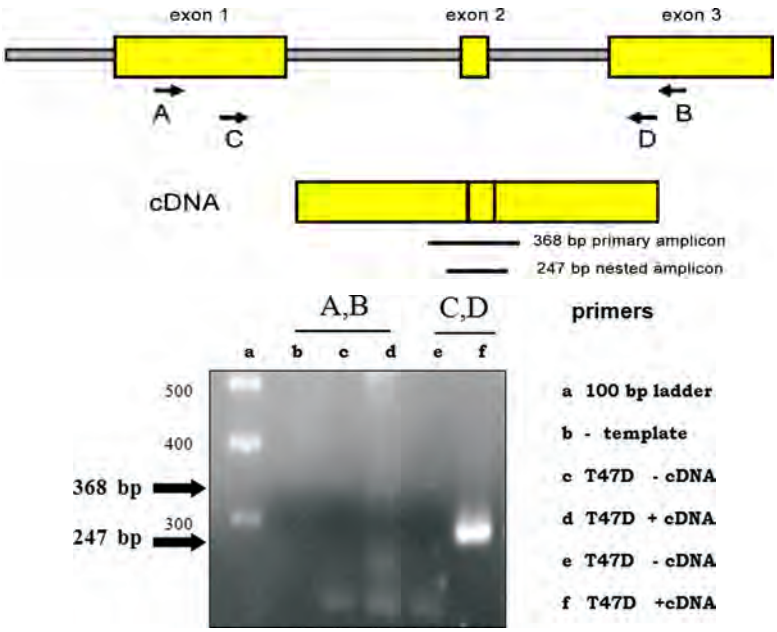


**Figure 1a: GnRH receptor mRNA expression in T47D cells:** Agarose gel displaying RT-PCR with two sets of exon spanning primers human GnRH receptor gene. Lanes b, c and d (primer A and B). Lanes e and f nested PCR products (primers C and D). Lanes b, c and e represent control reactions.

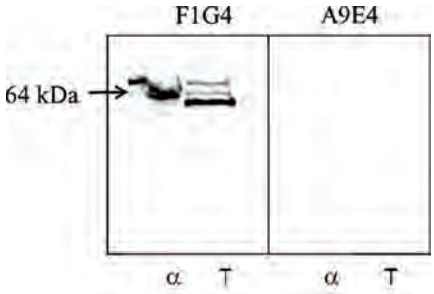


**Figure 1b: GnRH receptor protein expression:** Membrane protein preparations of αT3-1(α) andT47D (T) cells were separated by SDS-PAGE, blotted onto nitrocellulose membrane and probed with the anti-GnRH receptor mAb F1G4 (Left panel) followed by enhanced chemiluminescence. mAb A9E4 was used as isotype control (Right panel). The anti-GnRH receptor demonstrated 64 kDa band, whereas T47D, 3 bands of 68 kDa, 64 kDa, and 60 kDa were observed.

## GnRH receptor protein expression

To determine if the GnRH receptor mRNA was translated and expressed, Western blot analysis of membrane proteins of T47D cells was compared to those from αT3-1 mouse gonadotrope cells, used as the positive control, using the anti-GnRH receptor mAb, F1G4 (Karande *et al*., 1995). A single band of Mr 64 kDa was seen in the case of αT3-1 cells. However, in the case of T47D cells, three bands of Mr 68 kDa, 64 kDa, and 60 kDa were observed (Figure 1b). MAb A9E4 served as isotype control.

## Surface expression of GnRH receptors

The specificity of binding of the F1G4 antibody to the GnRH receptor was further established by immunofluorescence staining

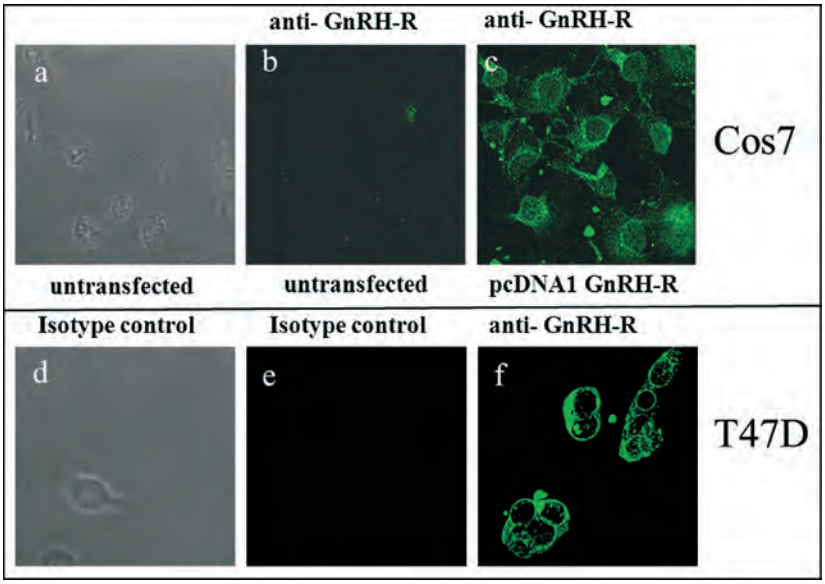


**Figure 1c: Cell surface expression of GnRH receptors:** Confocal immunofluorescence microscopic images of Cos-7 cells transfected with a human GnRH receptor expression vector (c) and untransfected cells (a, b) grown on slides, incubated with the mAb F1G4 followed by rabbit anti-mouse IgG-FITC conjugate. Phase contrast image of the untransfected cells are shown (a) in the upper panel. T47D cells grown on slides incubated with isotype control mAb A9E4 (e) and mAb F1G4 (f) followed by rabbit anti-mouse IgG-FITC. A phase contrast image of cells stained with isotype control mAb A9E4 (d) are shown in the lower panel.

followed by confocal microscopy of Cos-7 cells transfected with a mammalian expression plasmid, pcDNA1/amp GnRH-R encoding the human GnRH receptor gene under the control of the CMV promoter (Figure 1c). Staining with the antibody confirmed the expression of GnRH receptors on the cell surface of T47D cells. (Figure 1c). In an earlier study, using flow cytometry, 50–60% of T47D cells were shown to express GnRH receptors (Karande *et al*., 1995).

**Surface expression of GnRH receptors**

The specificity of binding of the F1G4 antibody to the GnRH receptor was further established by immunofluorescence staining followed by confocal microscopy of Cos-7 cells transfected with a mammalian expression plasmid, pcDNA1/amp GnRH-R encoding the human GnRH receptor gene under the control of the CMV promoter (Figure 1c). Staining with the antibody confirmed the expression of GnRH receptors on the cell surface of T47D cells (Figure 1c). In an earlier study, using flow cytometry, 50–60% of T47D cells were shown to express GnRH receptors (Karande *et al*., 1995).

**Effect of GnRH analogues on breast cancer cell proliferation**

Having established that T47D cells express GnRH receptor on their surface, the direct effect of GnRH receptor ligands on proliferation was assessed by treating these cells with GnRH/analogues (1–100 nM): the agonist Buserelin and the antagonist,

Cetrorelix, for two days in serum-free medium. Cetrorelix ~5 nM and Buserelin ~100 nM demonstrated 50% inhibition of proliferation of T47D cells. GnRH under these conditions did not show significant reduction in proliferation (Figure 2a). These molecules were toxic to the cells as studied by Trypan blue exclusion assay after incubation for 24–36 h (data not shown).

**Effect of GnRH analogues on growth factor stimulated c-fos gene expression**

T47D cells express epidermal growth factor (EGF) receptors and activate EGF receptor growth signaling pathways. A sensitive assay has been previously established (Beckers *et al*., 1997) to measure this activation. To ascertain whether the anti-proliferative effects of Cetrorelix may be due to inhibition of the classical growth factor signaling pathways, T47D cells were co-transfected with a vector expressing the luciferase reporter gene under the control of the c-fos promoter, pFL711, and a control vector expressing HSV-7 promoter driven Renilla luciferase gene, pRL-TK. The transfected cells were serum-starved for 72 h and pretreated with Cetrorelix and GnRH agonist [Trp$^6$] GnRH for 1 h prior to the addition of 100 nM of EGF for 10 min. After 6 h, the cells were lysed and luciferase activities were measured. EGF was able to elicit a three-fold increase in *c-fos* promoter activity (Figure 2b). However, neither Cetrorelix nor [Trp$^6$] GnRH at the concentrations (10, 100 and 1000 nM) tested abrogated the basal or the EGF stimulated *c-fos* promoter activity (Figure 2b). This suggested that the anti-proliferative effect of GnRH analogues does not involve perturbation of EGF signaling.

**Effect of GnRH analogues on cAMP production**

The direct effects of GnRH receptor occurs by the activation of effector proteins, such as PLCβ via the recruitment of G-proteins, specifically, G- q/11 subunit in pituitary cells and adenylyl cyclase via G-$\alpha_s$ subunits in rat pituitary organ cultures where cAMP is formed

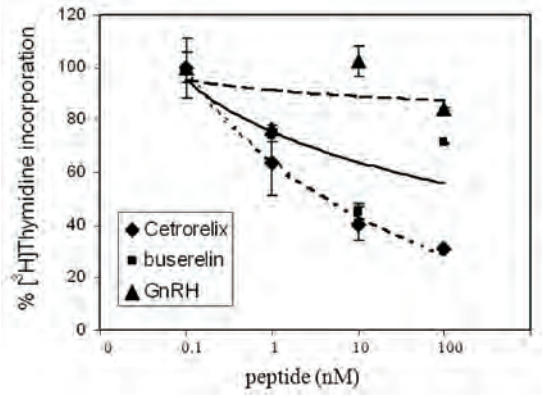


**Figure 2a:** Effect of GnRH and its analogues on T47D cell proliferation: Cells were treated with indicated concentrations of GnRH, Buserelin or Cetrorelix for 2 days. At the end of the treatment cells were incubated with [$^3$H]Thymidine (12 h), cells harvested onto glass fibre filters and the radioactivity measured. The results are expressed as percentage [$^3$H]Thymidine incorporated compared to untreated controls.

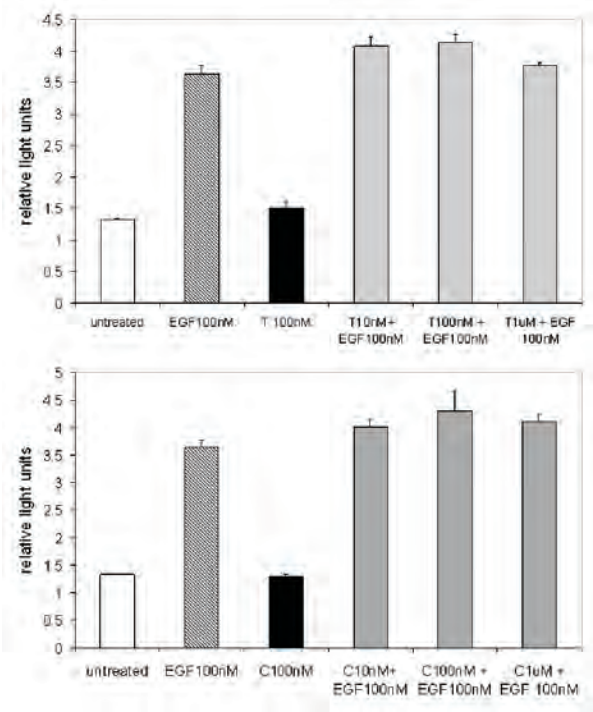


**Figure 2b: Effect of GnRH analogues on EGF-stimulated c-fos promoter activity:** T47D cells co-transfected with c-fos promoter-firefly luciferase reporter and HSV-TK-Renilla luciferase reporter, serum-starved (72 h), [Trp$^6$] GnRH (T) or Cetrorelix (C) (10–1000 nM) treated (20 min) and stimulated with 100 nM EGF(epidermal growth factor) for 10 min were lysed and luciferase activities measured 6 h post stimulation. Luciferase reporter activity is plotted as relative light units (ratio of firefly Luciferase activity to Renilla Luciferase activity) on the Y-axis.

in response to GnRH stimulation (Karande and Rajeshwari, 1999). Therefore, cAMP production in T47D cells in response to stimulation by GnRH and its analogues was measured by radioimmunoassay. None of the peptides tested were able to elicit increase in intracellular cAMP levels in T47D cells (Figure 3a). It is possible that other G-proteins such as $G\alpha_i$ that inhibits adenylyl cyclase may be recruited by GnRH receptor by as yet unknown mechanisms in an analogue or cell-type specific manner. The diterpene, forskolin (2.5 or 5 μM), is a constitutive activator of adenylate cyclase and T47D cells treated with forskolin leads to increased accumulation of cAMP. When cells were treated with cetrorelix for 20 min prior to forskolin addition, ~40–50% reduction in cAMP levels was observed with the antagonist (Figure 3b) at 1 M and 10 M. Pertussis toxin specifically ADP-ribosylates the adenylate cyclase inhibitory G-protein $G_i$ locking it in inactive GDP bound state relieving adenylyl cyclase of its inhibition leading to increased cAMP levels. When T47D cells were treated with 50 ng/ml of pertussis toxin, the inhibitory effect of cetrorelix (~25%) on forskolin-mediated cAMP production was completely abrogated. Pertussis toxin treatment alone during the 20 min period did not significantly increase basal levels of cAMP in T47D cells (Figure3c) nor did the toxin inhibit forskolin-mediated

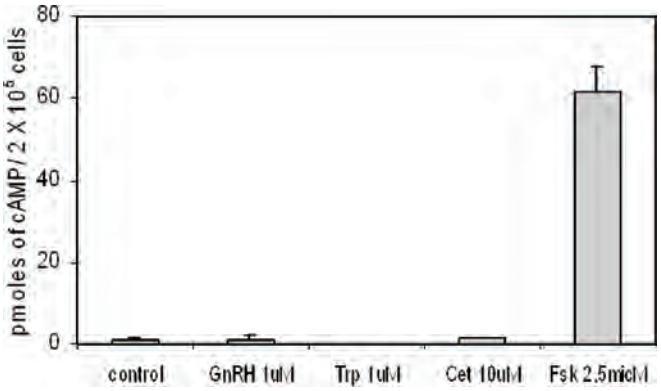


**Figure 3a: Effect of GnRH analogues on cAMP accumulation in T47D cells:** Cells plated in 24-well plates were incubated with 500 μM IBMX for 15 min at 37 °C prior to treatment with GnRH (1 μM), [Trp$^6$]GnRH (1 μM) and Cetrorelix (10 μM) or Forskolin (2.5 μM) in serum-free medium for 30 min. cAMP was extracted with 0.1 M HCl and measured by radioimmuno assay (RIA).

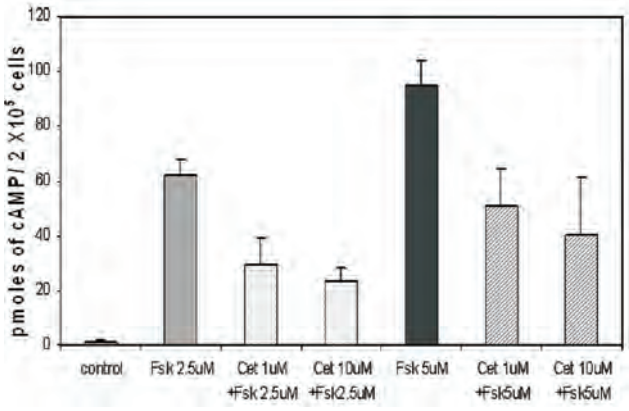


**Figure 3b: Effect of cetrorelix on forskolin-mediated cAMP accumulation in T47D cells:** cAMP levels as measured by RIA of cells pretreated with cetrorelix (1–10 μM) for 20 min prior to the addition of forskolin (2.5 μM and 5 μM) for 30 min.

increase in cAMP (data notshown), suggesting that $G\alpha_i$ is involved in cetrorelix-mediated effects on cAMP accumulation.

## DISCUSSION

We used the human breast cancer cell line, T47D as a model cell line to check for the presence of GnRH receptors and the effects of GnRH hormone and analogues on these cells. Human GnRH receptor specific exon spanning primers were employed to detect receptor mRNA by RT-PCR. A nested PCR demonstrated the specific 247 bp amplicon indicating low, regulated expression of the receptor mRNA (Figure 1a). Western blot analysis of membrane proteins of T47D cells showed the presence of multiple bands in the region of the reported molecular weight of the human GnRH receptor (Figure 1b). These bands may correspond to either differentially glycosylated or truncated forms of the receptor. The precise identity of these cross-

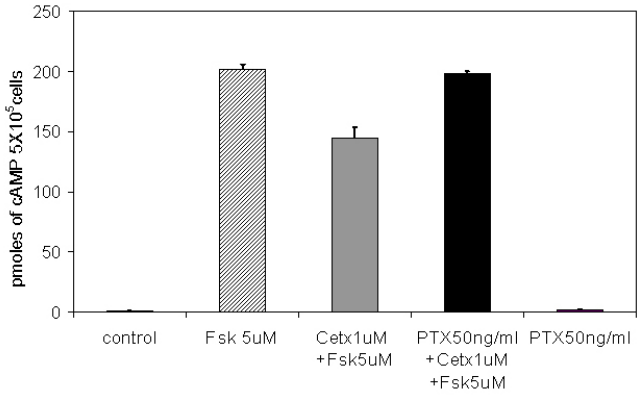


**Figure 3c: Effect of Pertussis toxin on Cetrorelix inhibition of cAMP levels in T47D cells:** Pertussis toxin (PTX) (50 ng/ml) was added to cells ($5 \times 10^5$ cells/ well) cultured in 12-well plates for 30 min prior to the addition of Cetrorelix for 20 min and subsequent addition of Forskolin (FSK) (5 μM) for 30 min. cAMP was estimated by RIA

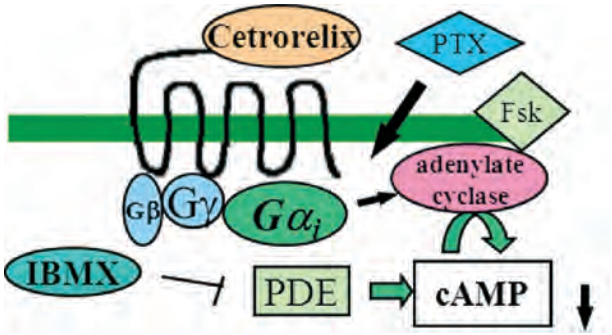


**Figure 3d: Scheme of factors involved in regulating cAMP levels in T47D cells:** GnRH receptor is coupled to the $G\alpha_{i/0}$ heterotrimeric G-protein. Upon cetrorelix-induced activation of the receptor, the $G\alpha_{i/0}$ protein gets activated leading to the inhibition of the adenylate cyclase. Pertussis toxin inactivates $G\alpha_{i/0}$

reactive species may indicate the role of GnRH receptors in extrapituitary tissues. A single band of 64 kDa was obtained in the case of the αT3-1 mouse gonadotrope cells, corresponding to previously reported human pituitary receptor (Karande *et al*., 1995). The F1G4 mAb was used previously to identify bands corresponding to this molecular weight in prostate cancer cell lines (Limonta *et al*., 1999). Immunostaining followed by fluorescence microscopy with the F1G4 mAb confirmed surface expression of GnRH receptors on T47D cells, and specificity of the antibody was established by detection of cell surface fluorescence on Cos-7 cells that do not express GnRH receptors, after transfection with a hGnRH receptor expression construct (Figure 1c). Flow cytometric analysis had previously revealed that 50% of the cells show specific staining for GnRH receptors (Karande *et al*., 1995).

Having confirmed the expression of GnRH receptors in T47D cells, the incorporation of [$^3$H]thymidine by these cells was carried out to study the role of GnRH in modulating proliferation. Treatment of the

cells with the native GnRH decapeptide did not stimulate DNA synthesis (Figure 2a). It is possible that we did not detect a minor effect, if any, because proliferation of the cells is highly deregulated as compared to normal cells. However, our studies showed an inhibition of T47D cell proliferation with analogues of GnRH. Cetrorelix brought about the maximum inhibition, up to 80%, at a concentration of 100 nM in T47D cells. Buserelin inhibited proliferation to a lesser extent up to 40% at 100 nM (Figure 2a). Both analogues, which are classified as agonist (buserelin), and antagonist (cetrorelix), based upon their action on pituitary cells behave in a similar way in the case of breast cancer cells. The data suggests that GnRH receptor mediated signaling in breast cells may be different from that seen in the case of the pituitary release of LH and FSH.

We tested whether GnRH analogues could interfere with signaling events mediated by growth factors as reported in other cell types (Emons *et al*.,1997a; Emons *et al*., 1998; Imai *et al*., 1996a; Imai *et al*., 1996b; Lee *et al*., 1991; Liebow *et al*., 1991; Moretti *et al*., 1996; Yano *et al*., 1994). Induction of *c-fos* gene expression is a well-known response to a number of growth factors and hormones. We measured *c-fos* promoter response to EGF with a luciferase reporter assay. However, our results show that under conditions in which EGF brought about a three-fold increase in *c-fos* promoter activity (Figure 2b) and increased proliferation (data not shown), [$Trp^6$]GnRH or Cetrorelix had no effect on c-*fos* promoter activity either in the presence or absence of EGF (Figure 2b). Our studies indicate that GnRH analogues have no effect on EGF-induced growth signaling in T47D breast cancer cells.

GnRH signaling in pituitary cells at GnRH hormone concentrations required for LH and FSH release predominantly occurs through the Gq/11-PLCβ pathway (Emons *et al*. 1997a). However, as our group has shown previously, it also leads to the formation of cAMP (Karande and Rajeshwari, 1999). Activation of adenylyl cyclase has been demonstrated in response to GnRH in recombinant insect cells expressing GnRH receptor (Delahaye *et al*., 1997). The GnRH receptor has also been shown to couple to other G-proteins when these were over-expressed in GGH3 cells. Buserelin (a GnRH agonist) stimulated increased cAMP release in $Gs_{\alpha}$ cDNA-transfected GGH3 cells (Stanislaus *et al*., 1998). In prostate cancer cells (Limonta *et al*., 1999) and on female reproductive tract tumors (Imai *et al*., 1996c), GnRH receptor seems to be coupled to the $G\alpha_i$ protein-cAMP signal transduction pathway, rather than to the Gα (q/11)-phospholipase C signaling system. This indicates that GnRH receptor may possess differential G-protein coupling specificities in differing cellular contexts. Increased cAMP levels are usually mediated via $G\alpha_s$-proteins, whereas reductions in cAMP levels are most likely due to activation of $G\alpha_{i/o}$-proteins. In our hands none of the GnRH analogues tested triggered cAMP formation above basal levels (Figure 3a) suggesting that GnRH receptor in T47D cells is not, at least in the case of these ligands, coupled to the adenylate cyclase

stimulatory G-protein, $G\alpha_s$. Cetrorelix brought about maximum inhibition of proliferation in T47D cells (Figure 3b), and this analogue also inhibited forskolin stimulated cAMP accumulation in a dose-dependent manner (Figure 3c). It is pertinent to mention here however that significant inhibition was seen only at higher concentration of the analogue. Moreover, the inhibition of forskolin stimulated cAMP by cetrorelix in T47D cells was found to be Pertussis toxin-sensitive (Figure 3c, 3d). Overall, these results indicate that the GnRH receptor engaged by Cetrorelix is coupled to the $G_{i/0}$ heterotrimeric G-protein in T47D cells (Figure 3d). Modulation of intracellular cAMP levels triggered by growth factors may be a possible route by which cetrorelix exerts antiproliferative effects on breast cancer cells. There have been reports that estrogenic hormones that induce proliferation at concentrations between 1 pM and 1 nM in T47D of breast cancer cells (Chalbos *et al*., 1982) and at approximately 1 nM for MCF7 cells (Weichselbaum *et al*., 1978), increase cAMP in target breast cancer MCF7 cells in culture (Aronica *et al*., 1994). Increases in intracellular cAMP by estrogens are evoked by very low concentrations of estradiol (half maximal at 10 pM) and by other physiologically active estrogens and anti-estrogens. The increases in cAMP result from enhanced membrane adenylate cyclase activity by a mechanism not involving genomic actions of the hormones as they are not blocked by inhibitors of RNA and protein synthesis (Aronica *et al*., 1994). It was also seen that the estrogen-stimulated levels of cAMP are sufficient to activate transcription from cAMP response element containing genes and reporter plasmid constructs (Aronica *et al*., 1994). Further studies will address the role of GnRH receptor in Cetrorelix inhibition of estrogen induced proliferation in breast cancer and the role of cAMP pathways in mediating these effects.

## CONCLUSIONS

The studies presented demonstrate that GnRH receptors are present on the T47D breast cancer cell line and that both antagonist as well as agonist of GnRH inhibits proliferation of these cells. The inhibition may not be via the suppression of EGF signaling and subsequent c-fos promoter activity, but via inhibition of cAMP accumulation. This effect was pertussis toxin sensitive suggesting that in T47D cells, GnRH receptors are coupled with the $G_{i/0}$ heterotrimeric G-protein, a situation different from that seen in the case of the pituitary cells where the receptor is well known to couple to the $G_{q/11}$ isoform.

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## CONFLICT OF INTEREST

The authors claim no conflict of interest.

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Electronic supplementary material to support and enhance your scientific research is accepted as supplementary file. Supplementary files offer the Author additional possibilities to publish supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article. In order to ensure that your submitted material is directly usable, please ensure that data is provided in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file.

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# About School of Science

School of Science was started in 2007 with a view to provide undergraduate and post graduate students an opportunity to be a part of the unique learning methodology of the university, which lays emphasis on academic excellence combined with industry oriented training. With the boom in information technology and more and more sophistication in instrumentation techniques, there is now a very thin dividing line between the various disciplines of science. Therefore, there is a greater need for flexibility in scientific thought as well as training manpower on an interdisciplinary plane. With this thought in view, the SVKM's NMIMS introduced, highly innovative and unique interdisciplinary courses at the School of Science from the academic year 2007-2008. The goal of the School of Science is to be a Center of Excellence in the domain of Pure and Applied Science by providing quality education and research.

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# Courses Offered

## Doctoral Programs

### Ph.D. Biological Sciences

Full time: One year course work (three trimesters) + Minimum three years of research work.

### Ph.D. Chemical Sciences

Full time: One year course work (three trimesters) + Minimum three years of research work.

## Integrated Masters-Doctoral Programs

### Integrated M.Sc.-Ph.D. Biological Sciences

Full time: Two years M.Sc. course work (six trimesters) followed by minimum three years of doctoral research work.

### Integrated M.Sc.-Ph.D. Chemical Sciences

Full time: Two years M.Sc. course work (six trimesters) followed by minimum three years of doctoral research work.

## Masters Programs

### M.Sc. in Biological Sciences

Full time: Two years (four trimesters of course work + six months of research project in reputed institutions.)

### M.Sc. in Chemical Sciences

Full time: Two years (four trimesters of course work + six months of research project in reputed institutions.)

### M.Sc. in Statistics

Full time: Two years (Six trimesters including six months of project work and summer internship).

### Master of Physiotherapy

Full time: Two years (Six trimesters) [In collaboration with **Dr. Balabhai Nanavati Hospital**, Mumbai, India]

## Post-Graduate Diploma Programs

[In collaboration with **Asian Heart Institute and Research Centre**, Mumbai, India]

**Physician Assistant**
Full time: Two years

**Operation Theatre Technician**
Full time: One year

**Non-Invasive Cardiology Technician**
Full time: One year

**Central Sterile Supply Department Technician**
Full time: One year

## Diploma Program

[In collaboration with **Asian Heart Institute and Research Centre**, Mumbai, India]

**Clinical Research**
Part time: One year

## Certificate Courses

**Molecular Medicine/Molecular Oncology**
Part time: Six months course for medical professionals

**Advanced Course in Clinical Data Management**
Part time: Three months [In collaboration with **C. B. Patel Research Centre**, Mumbai, India]

# Salient Features

- Research constitutes a major thrust in all the courses offered at the School
- Courses oriented to fulfill needs/demands of Research Institutions/Industry

# Thrust Areas

Cell Biology, Stem Cell Biology, Molecular Oncology, Reproductive Biology, Microbiology, Immunology, Pharmacology, Phytochemistry, Nanosciences, Applied Chemistry, Colloidal Chemistry and Applied Statistics

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