BIOMARKERS
6.1 Background

6.1.1 Immunity and Cancer

Cancer remains as one of the leading cause of death worldwide. Over the last decade, molecular discovery of tumor antigens as well as new findings in basic immunology has led to novel options to develop active immunotherapeutic approaches for prevention and treatment of cancer. The immune response is the body’s way of defending itself against disease. Immune defense against tumors is mediated through both antigen-specific and nonspecific immune mechanisms. Nonspecific immune responses are procured by cells of the macrophage and Natural Killer (NK) cell lineage and/or by soluble factors such as inflammatory cytokines. Nonspecific immunostimulation have been applied for treatment of cancer. The functioning of the antigen specific immune system is based on a division of labor between T cells and antibody-producing B cells (Zinkernagel and Hengartner, 2001) (Figure 40).

* Figure 40: Mediators of immune defence*
Evidence from a number of different investigations suggests a possible role of the immune system to treat cancer. A positive correlation between tumor infiltrating T lymphocytes and patients survival has been observed (Wada et al., 1998; Zhang et al., 2003). Spontaneous tumor-specific T cell responses occur in individuals with premalignant lesions (Dhodapkar et al., 2003; Suzuki et al., 2005; Garbe et al., 2006) and have been found in patients with a variety of different tumors (Nagorsen et al., 2003; Korangy et al., 2004).

6.1.2 Tumours escapes immunity

Tumors have developed a number of different strategies to escape immune surveillance such as (i) loss of tumor antigen expression, (ii) MHC down regulation, (iii) expression of Fas-L, which can induce apoptosis in activated T cells, (iv) secretion of VEGF and other cytokines such as IL-10 or TGF-β (Gabrilovich et al., 1996; Ferrara et al., 2003; Dunn et al., 2004). Other mechanisms include the generation of regulatory T cells and myeloid suppressor cells (Ormandy et al., 2005; Zou, 2006; Kusmartsev and Gabrilovich, 2006). The requirements for an immune-based strategy against cancer are the induction of an effective tumor specific immunity in a way that will break tolerance to the tumor and generate anti-tumor immunity.

6.1.3 Autoantibodies against Tumor associated antigens

Many studies demonstrated that the immune system can recognize the antigenic changes in cancer cells, and further develop autoantibodies against these cellular components that have been described to be aberrantly expressed in cancer and generally been called as tumor-associated antigens (TAAs). Therefore, these cancer-associated autoantibodies might be considered as “reporters” from the immune system, to identify the antigenic changes in cellular proteins involved in the transformation process (Tan, 2001; Old and Chen, 1998; Tan and Zhang, 2008). The mechanisms underlying the emergence of autoantibodies and the regulation of their production are not completely understood. Some reports have suggested that this immune response is due to a self-immunization process linked to increased immunogenicity of TAAs during malignant transformation (Sheever et al., 1995). Although this research area is advancing, a number of fundamental questions remain unresolved.
6.1.4 Cellular components that induce autoantibodies

One of the most extensively studied TAAs is p53, a tumor suppressor protein. Autoantibodies to p53 in cancer were first reported in 1982 (Crawford et al., 1982), and since then there have been numerous reports confirming and extending this finding (Soussi, 2000). The types of cellular proteins that induce autoantibody responses are quite varied and include oncogene products such as HER-2/neu (Disis et al., 1997), onconeural antigens (Keene, 1999), differentiation-antigens such as tyrosinase and the cancer/testis antigens (Stockert et al., 1998), and antiapoptotic proteins such as surviving (Ambrosini et al., 1997), LEDGF (Danieli et al., 2005), etc. A highly informative study showed that in lung tumors containing several types of p53 gene mutations, including missense, stop codon and frameshift mutations, only the missense p53 mutations, with overexpression of a protein that altered function and increased protein stability, correlated with autoantibody production (Winter et al., 1992). In case of p62/IMP2, a fetal protein absent in adult tissues, the presence of autoantibodies relates to abnormal expression of p62 in tumor cells (Zhang et al., 1999; Lu et al., 2001).

6.1.5 Autoantibodies in immunodiagnosis

In recent years, the potential utility of TAA-autoantibody systems as early cancer biomarker tools to monitor therapeutic outcomes or as indicators of disease prognosis has been explored. The major reason is that these antibodies are generally absent, or present in very low titers, in normal individuals and in non-cancer conditions (Tan and Zhang, 2008). Their persistence and stability in the serum of cancer patients is an advantage over other potential markers, including the TAAs themselves because as some of which are released by tumors but rapidly degraded or are cleared after circulating in the serum for a limited time (Anderson and LaBaer, 2005). Furthermore, the widespread availability of methods and reagents to detect serum autoantibodies facilitates their characterization in cancer patients and assay development.
The definition of what constitutes a TAA is a major issue in the field of cancer immunodiagnosis. It is erroneous to include all cellular antigens identified by autoantibodies in cancer sera as TAAs since some autoantibodies may exist in conditions that pre-date malignancy. Failing to recognize the likelihood of premalignancy circulating antibodies would result in the inclusion of many antigens erroneously as TAAs, especially if serum drawn at one time point from a cancer subject was used to characterize the antigens since this might include both cancer-related and unrelated antigens. Some of the cellular proteins identified by cancer autoantibodies were initially of unknown function but eventually were shown to be involved in tumorigenesis pathways. The identification and characterization of two novel TAAs p62 and p90 are examples of this kind of studies.

6.1.6 Vaccines for cancer

Vaccines preventing infections of oncogenic viruses are today well established, for example, for hepatitis B (HBV) or human papilloma virus (HPV) associated cancers, and have a great benefit in human and veterinary medicine (Plymoth et al., 2009; Schiller and Lowy, 2006; Suzich et al., 1995; Bergman et al., 2006). For virus-independent cancerogenesis there is the need to directly target tumor-associated antigens (TAAs). Active immunotherapy could lead to specific and sustained immunity against tumors, thereby counteracting already ongoing malignant growth. Such therapeutic anticancer vaccines may be constituted of patient-specific cellular material, TAAs and derivatives thereof (Bilusic and Madan, 2011), or DNA encoding TAAs (Bergman et al., 2006), all aiming at induction of immunologically mediated tumoricidal or tumorstatic mechanisms. Principally, tumor vaccines might have the capacity to combat already established malignant disease, but it has to be faced that the success of immunological strategies, like of any other anticancer therapy, will indirectly correlate with the tumor load and stage. The ideal scenario of a tumor vaccine might be the setting of minimal residual disease where, at low numbers of aberrant or cancer stem cells, the actively induced immune mechanisms would have a fair chance to prevent tumor relapse through reduction of circulating tumor cells
and micrometastases (Karbassi et al., 2005). In addition, prophylactic TAA-based vaccines may be conceivable in cases of hereditary predisposition.

6.1.7 Passive versus active antibody therapies in oncology

Among all immunological strategies, antibody therapies are today state of the art (and pharmaceutical blockbusters) in oncology, with classics such as trastuzumab (Herceptin®), cetuximab (Erbitux®) and rituximab (Rituxan®) being applied worldwide in cancer patients with a doubling market within the last 5 years (Business insight, 2006). Therefore, passive immunotherapies are generally much more advanced in clinical oncology than vaccines, except the truly prophylactic anticancer vaccines preventing oncogenic virus infections. One great advantage of passive immunotherapy is that it may be discontinued at any point of time. However, even though today’s antibodies are chimeric, humanized or fully humanized, side effects can regularly be observed in clinics. The use of antihistamines or corticosteroids control and prevent side effects (Siena et al., 2010; Schwartzberg et al., 2008).

It is obvious that vaccines, in contrast, induce complete self-antibodies without these immune-mediated risks. Eventual induction of autoimmunity as a side effect nourishes sceptic criticism against TAA-based cancer vaccines, but is also observed in other immunotherapeutic approaches such as passive antibody therapy and trials with cytokines as immunomodulators (Amos et al., 2011). Evidently, many cancer antigens are self-antigens and are also expressed by healthy cells of the body, although to a lesser extent (Fin et al., 2010). There is also evidence that the extent of post-translational modification may contribute to the immunogenicity of cancer antigens such as mucin 1 (MUC1) (Ryan et al., 2010; Farkas et al., 2010). Therefore, the risk of autoimmunity is justified and has to be weighed against the threats of the malignancy in the individual patient. The phenomenon that other anticancer therapies also regularly lead to autoimmunity (Capsi et al., 2008) is less recognized, possibly because it needs longer survival times to become clinically relevant.
(Correale et al., 2008). However, it is easy to explain from an immunological point of view: radiotherapy, chemotherapy and biologicals, including passive antibody therapies (Towns et al., 2008), lead to destruction of tumor cells often associated with oxidative stress. In consequence, modified self-antigens are liberated during cancer destruction, taken up by antigen-presenting cells and may to various degrees lead to breaking of tolerance and autoimmunity against many membranous and intracellular antigens that occur in healthy cells as well (Mcbride et al., 2004). Autoimmune gastritis, thyreoiditis (Smyth et al., 1998; Kilbane et al., 1998), vitiligo or uveitis (Bouwhuis et al., 2011) may be typical late complications, depending on the origin of the tumor. Therefore, autoimmunity may be a common risk during anticancer therapies. So, highly specific autoimmunity should be the aim of cancer vaccines (Pardoll et al., 1999), and has to be associated with prolonged survival (Gogas et al., 2006). Needless to say, that the careful selection of the appropriate TAA is a condition for successful vaccination strategies. Furthermore, the specific epitope of a tumor antigen may critically determine the biological outcome. Data from animal studies indicate that upon careful selection there may be the chance to vaccinate against cancer without inducing autoimmunity to healthy tissues (Lute et al., 2005; Greiner et al., 2002; Wang et al., 2010).

6.1.8 Antibody therapies: where do we stand?

Another argument in favour of vaccines comes from the economic point of view: passive immunotherapies are characterized by restricted duration of action based on the half-life and elimination rate of the applied antibodies (Petkova et al., 2006). The latter is dependent on the isotype, and may be improved by engineering to adapt fit to Fc-receptors (Shields et al., 2001). For instance, trastuzumab is applied at 2–8 mg/kg bodyweight (Hudis, 2007), thus achieving dosages between 140 and 560 mg in an average 70 kg patient (Matter-Walster et al., 2010). For bevacizumab (Avastin®) even higher amounts, 10 mg/kg – that is, 700 mg in the standard patient are applied every 2 weeks (Giantonio et al., 2007). These huge amounts make antibody therapies the most cost-intensive treatment options available.
By contrast, classical vaccines are expected to cost less and could help to overcome these monetary limitations. An example is the recently presented US FDA-approved prostate cancer vaccine sipuleucel-T (Provenge®) (Kanloff et al., 2010). Sipuleucel-T improves survival, although by only approximately 4 months. The complex protocol results in heavy costs of over US$90,000 for a three-step therapy. Similar autologous strategies are attempted successfully for other tumor entities, such as melanoma (Ramano et al., 1997). However, tumor vaccines in the classic sense are economically attractive concepts for health authorities and, consequently, accessible for a larger number of eligible patients.

Since the invention of monoclonal antibody technology (Kohler and Milstein, 1975), the overwhelming majority of antibodies produced by hybridoma technology belong to the IgG class. This also holds true for oncologically applied immunoglobulins, which are all IgG, whereas all other isotypes are more or less neglected (Reichert and Wenger, 2008). This means that antibody therapies today rely on a very restricted effector cell panel harboring IgG receptors. For instance, IgA (Dechant et al., 2007; Otten et al., 2005) and IgE anticancer antibodies may have excellent tumoricidic properties (Fu et al., 2008; Karagiannis et al., 2009; Jensen-Jarolim et al., 2008). By contrast, vaccines produce polyclonal antibody responses that simultaneously take advantage of multiple receptors and different types of highly specific effector cells. However, the resulting immune response can be biased towards Th1, Th2 or Th3 by usage of different adjuvants and the choice of route (Bunner et al., 2010). It has been recently shown that subcutaneous vaccination with HER-2 mimotope peptides resulted in IgG antibodies in BALB/c mice (Riemer et al., 2004), whereas orally administered peptides induced HER-2-specific IgE, harbouring tumoricidic properties (Riemer et al., 2007).

Today a great number of cancer vaccine trials between Phase II and III (Cecco et al., 2010) are ongoing and registered in the National Cancer Institute database (www.cancer.gov/clinical trials). The clinically tested antigens specifically derived from cancer cells include whole tumor cells, pulsed dendritic cells (DCs) or Langerhans cells, TAAs in native or modified form, proteins, carbohydrates,
vaccines, glycoproteins or glycopeptides, gangliosides, RNA as well as DNA and B- or T-cell epitope peptides. The usage of whole tumor antigens may be hampered by the fact that besides beneficial specificities, tumor promoting antibodies may also be induced (Reichert and Wenger, 2008; Harwerth et al., 1993; Hurwitz et al., 1995; Yip et al., 2001). Therefore future studies should aim in developing epitope-specific immunization.

6.1.9 Objective

In this regard the present study was carried out to determine the immunogenic TAAs from the study population and to characterize them by MALDI-ToF analysis. The identified proteins were subjected for epitope prediction that may be utilized for the development of epitope specific vaccine in future research.
6.2 Materials and Methods

6.2.1 Sample collection
A total of 39 fresh ovarian tissue samples (as per section 5.3.1 and 5.3.2) were collected and used for the extraction of proteins. Out of the 39 ovarian tissue samples, 30 were from ovarian cancer patients and 9 from healthy controls. Out of the 30 ovarian cancer tissues, 24 were of invasive type (12 serous, 6 endometrioid and 6 mucinous) and 6 were of borderline tumours (all serous types). The tissue samples were procured during laparotomies and transported to the laboratory in ice cold PBS (1X) and stored at -20°C until use.

6.2.2 Extraction of proteins from tissue samples
For each sample, 0.2 g of tissue was ground with a precooled mortar and pestle using 1 ml of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-Cl, pH 8.5). The samples were centrifuged for 10 min at 12,000 rpm to remove particulate materials. The supernatant thus obtained served as protein source. Protein concentrations were determined in duplicate by BCA method (Sigma-aldrich).

6.2.3 1D Gel electrophoresis
The proteins were then solubilised in 2X SDS-PAGE sample buffer (0.125 M Tris-Cl (pH 6.8), 5mM EDTA, 2% SDS, 0.1% bromophenol blue, 1% 2-mercaptoethanol) by heating at 100°C for 10 min and then centrifuged at 12,000 g for 10 mins. The supernatant thus obtained was loaded on a 12% polyacrylamide gel and electrophoresed according to Laemmli, 1970.

6.2.4 Western blotting
After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose (0.45-mm) membranes (Whatman, Maidstone, U.K.) with Tris-glycine buffer containing 20% (v/v) methanol. Each membrane was then blocked with TBS-
0.1% tween 20 (TTBS) containing 4% (w/v) non-fat milk powder. The membranes were washed thrice with TTBS and then incubated with patients’ pooled plasma for 1h at 37°C. Following incubation the membranes were washed thrice with TTBS and incubated for another 1h at 37°C with antihuman-IgG–peroxidase conjugate (Sigma). The membranes were washed thrice and the blots were developed with substrate solution (25 mg of diaminobenzidine, 30 ml H₂O₂ in 50 ml TBS). The blots were then rinsed several times in de-ionized water, to stop the reaction.

6.2.5 Mass spectrometry and protein identification
6.2.5.1 In-gel digestion of protein spots with trypsin
The spots suspected to have tumour associated antigens (TAAs) were excised manually and mass spectrometry analysis was carried out. The protein spots were digested following the standard protocol (Shevchenko et al., 2006). Gel plugs were subjected to reduction with 10 mM DTT in 50 mM ammonium bicarbonate and alkylation with 55 mM iodoacetamide in 50 mM ammonium bicarbonate. The pieces of gel were then rinsed with 50 mM ammonium carbonate and acetonitrile and dried under a stream of nitrogen. Modified porcine trypsin at a final concentration of 13 ng/ml in 50 mM ammonium carbonate was added to the dry pieces of gel and digestion was allowed to proceed at 37°C for 6 h. Finally, 0.5% trifluoroacetic acid was added for peptide extraction. For peptide mass fingerprinting and the acquisition of TOF spectra an aliquot of the above digestion solution was mixed with an aliquot of alpha cyano 4-hydroxycinnamic acid in 33% aqueous acetonitrile and 0.1% trifluoro acetic acid. This mixture was deposited onto a 600 µm Anchor Chip MALDI probe and allowed to dry at room temperature.

6.2.5.2 MALDI-TOF analysis
Peptide mass fingerprint was measured on an Opti-TOF 384 well insert (Applied Biosystems/MDS Sciex, Foster City, CA) with 0.3 µl of 5 mg/ml alpha-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St. Louis MO) in 50% CH₃CN, 50% 0.1% trifluoroacetic acid. Crystallized samples were washed with cold 0.1% trifluoroacetic acid and analyzed by Applied Bio systems 4800 MALDI TOF/TOF Proteomics.
Analyzer. An initial MALDI MS spectrum was acquired for each spot (400 laser shots per spectrum) and a maximum of 15 peaks with a signal-to-noise ratio of greater than 20 were automatically selected for MS/MS analysis (1,000 shots per spectrum) by post-source decay or by collisionally-induced dissociation using air at a pressure of 5e-7 Torr. Peak lists from the MS/MS spectra were submitted for database similarity searching using Protein Pilot (vs. 2.0, Rev. 50861; Applied Biosystems). The molecular function and biological process involvement of the identified proteins were assigned according to the gene ontology database (http://www.geneontology.org) and the Swiss port/uniprot database (http://beta.uniprot.org).

6.2.6 Prediction of B-cell epitope

Protein sequences of the identified immunogenic proteins were obtained from NCBI and were subjected for BCPreds (El-Manzalawy et al., 2008). BCPred identifies common B-cell epitopes. The epitopes with BCPreds score of >0.8 and VaxiJen score >0.4 were predicted as highly immunogenic epitope that can be used for the preparation of vaccines.
6.3 Results and Discussion

6.3.1 Extraction of proteins from tissue samples

Proteins were extracted from 39 ovarian tissues and separated on a 12% polyacrylamide gel. The proteins were quantified by BCA method and equal concentration of protein samples were loaded in alternating order of control and case samples (Figure 41).

*Figure 41: Protein profile of cancerous and normal ovarian tissues*

Lane 1: CAO1; Lane 2: CON1; Lane 3: CAO2; Lane 4: CON2; Lane 5: CAO3; Lane 6: CON3; Lane 7: CAO4; Lane 8: CON4; Lane 9: CAO5; Lane 10: CON5; Lane 11: CAO6; Lane 12: CON6; Lane 13: CAO7; Lane 14: CON7; Lane 15: CAO8
6.3.2 Western blotting

The separated proteins were transferred to nitrocellulose membrane and the proteins were probed with pooled plasma samples. As shown in figure 42, immunoblot analysis of the protein preparation of control and case ovary tissues with patients’ plasma revealed around 4 immunodominant antigenic spots. Matching of the immunoblot with their homologues one dimensional CBB stained gel localized the antigenic spots at 19.3 kDa, 51.5 kDa, 62.1 kDa and 150 kDa. The healthy control tissues probed with plasma did not reveal such bands, thus envisaging the proteins as immunodominant. The protein of molecular weight 62.1 kDa was found to be expressed as immunogenic in all ovarian carcinoma cases irrespective of the stage and histology type. The protein of molecular weight 51.5 kDa and 19.3 kDa are expressed during stages III-IV (Figure 42).

Figure 42: Immunoblot analysis of ovarian tissue proteins probed with patients’ plasma
6.3.3 Mass spectrometry and protein identification

The highly immunodominant protein bands were excised from the CBB stained gel and subjected for MS analysis. The proteins' identities including its theoretical and experimental molecular weights, sequence coverages and MASCOT score was noted. The sequence coverage is the fraction of the complete protein sequence identified and MASCOT score is given as $S = -10 \times \log(P)$, where $P$ is the probability that the observed match would be a random event. MASCOT score values higher than 56 are considered to be significant ($P < 0.05$). The spots identified corresponds to four different proteins that are considered to be important tumor antigens (Figure 43 & Table 27).

Table 27: Details of the proteins identified by MALDI-TOF analysis

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the protein</th>
<th>Molecular weight (kDa)</th>
<th>Matches</th>
<th>MASCOT score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Testis specific antigen</td>
<td>62.1</td>
<td>15</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td>Gremlin-2</td>
<td>19.3</td>
<td>11</td>
<td>54</td>
</tr>
<tr>
<td>3</td>
<td>C16Orf88</td>
<td>51.5</td>
<td>18</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>Ceruloplasmin</td>
<td>150</td>
<td>14</td>
<td>53</td>
</tr>
</tbody>
</table>
6.3.4 Characteristics of the proteins identified

6.3.4.1 Gremlin-2

The gremlin-2 is a protein that belongs to DNA domain family which contains cysteine knots. Gremlin-2 is an important cytokine that inhibits the activity of BMP2 and BMP4 in a dose dependant manner. Antagonised BMP4-induced suppression of progesterone production in granulose cells. Thus the biological function of gremlin-2 is in BMP signalling pathway and its molecular function is cytokine activity.

The Drm (also known as gremlin) is a 184-aa protein initially identified through differential screening as a transcriptional down-regulated gene in v-mos-transformed rat embryonic fibroblasts (Topol et al., 1997), belongs to the Dan family of secreted glycosylated proteins (Topol et al., 2000; Pearce et al., 1999), which contains a highly conserved cysteine knot domain shared by the TGF-β superfamily, PDGF, nerve growth factor, and other secreted proteins (Isaacs, 1995). Drm and Dan regulate early development (Capdevilla et al., 1999; Dionne et al., 2001; Khokha et al., 2003; Zuniga et al., 1999), tumorigenesis (Topol et al., 1997; Chen et al., 2002; Hanaokae et al., 2000), and renal pathophysiology (Lappin et al., 2000).

Gremlin gene encodes a member of the bone morphogenic protein (BMP) antagonist family. Like BMPs, BMP antagonists contain cystine knots and typically form homo- and heterodimers. The cerberus and dan subfamily of BMP antagonists, to which this gene belongs, is characterized by a C-terminal cystine knot with an eight-membered ring. The antagonistic effect of the secreted glycosylated protein encoded by this gene is likely due to its direct binding to BMP proteins. As an antagonist of BMP, this gene may play a role in regulating organogenesis, body patterning, and tissue differentiation. In mouse, this protein has been shown to relay the sonic hedgehog signal from the polarizing region to the apical ectodermal ridge during limb bud outgrowth (Michos et al., 2004).

Several studies had previously shown that most tumor-derived cells fail to express Drm (Topol et al., 2000) and that in fibroblasts Drm expression is inhibited following oncogene-induced transformation (Topol et al., 1997). Human Drm maps to chromosome 15q13-q15, within a region whose loss is associated with metastatic breast cancer and other metastatic carcinomas (Wick et al., 1996). These properties
suggested that Drm might play an inhibitory role in cell transformation or tumorigenesis. They also demonstrated that overexpression of Drm in the tumor-derived cell lines Daoy (primitive neuroectodermal) and Saos-2 (osteoblastic) significantly inhibited tumorigenesis and provided evidence that Drm can function as a novel transformation suppressor and suggested that this may occur through its affect on the levels of p21\textit{Cip1} and phosphorylated p42/44 MAPK (Chen et al., 2002; Chen et al., 2004). Recent publication also demonstrated that gremlin mRNA is expressed in non-malignant epithelial cells and lost in many human cancer cell lines via promoter methylation (Suzuki et al., 2005). Similar finding is also reported by other group.

On the contrary, our experiments showed that GREM-2 was overexpressed in the cancerous ovarian tissues. Our result has been supported by a previous work where PIG-2 which is identical to GREM1 has been over expressed in various human tumors including carcinomas of the cervix, lung, ovary, kidney, breast, colon, pancreas and sarcoma. Moreover they have also reported a down regulation of PIG-2 in diversified human normal tissues. The results of the present study thus suggest GREM-2 may play a fundamental oncogenic role in ovarian tissues. However, it is unknown how GREM-2 contributes to the cellular and biochemical mechanisms of human tumorigenesis.

We hypothesize that Wnt may activate the BMP inhibitor Gremlin 2 in nearby fibroblasts, thereby blocking target BMPs from promoting differentiation and loss of self-renewal capacity thus enhancing tumourous growth (Figure 44).

\textit{Figure 44: Hypothesis to prove the role of Gremlin-2 in carcinogenesis}
The sequence of the gremlin-2 protein analysed by VaxiJen v2.0 server gave a score of 0.6317 showing the protein as antigenic in nature. The BCPreds predicted the epitopes for B-cell response. The epitopes with VaxiJen scores >0.4 were considered as antigenic. Finally 4 peptides were predicted as epitopes of B-cell response (Table 28).

### Table 28: Predicted epitopes of B-cell response for Gremlin-2 protein

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<thead>
<tr>
<th>1</th>
<th>11</th>
<th>21</th>
<th>31</th>
<th>41</th>
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<td>EEEEEE</td>
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<tr>
<td>VVTERKYLKSDWCKETQPLRQTVEEGCRSRTLNRFCYGQCN5SFYIPRHVKKEESFPQSC120</td>
<td>EEEEEE</td>
<td></td>
<td></td>
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</tbody>
</table>

S.Shanmughapriya, Department of Biochemistry, Bharathidasan University
Genetic, molecular and microbiological basis for ovarian carcinoma from a low incidence population

S.Shanmughapriya, Department of Biochemistry, Bharathidasan University

6.3.4.2 Testis antigen 62

The testis specific antigen 62 contains Leu-X-X-Leu-Leu motif and belongs to the coiled coil repeat family. The protein functions as nuclear receptor co-activator that can preferentially enhance estrogen receptor ESR1 and ESR2 transactivation. It also modulates progesterones/PGR, glucocorticoid/NR3C1 and androgen receptor/AR transactivation. The subcellular localization of the protein is cytoplasm or nucleus. The sequence of the testis specific antigen 62 protein analysed by VaxiJen v2.0 server gave a score of 0.6135 showing the protein as antigenic in nature. The BCPreds predicted the epitopes for B-cell response. The epitopes with VaxiJen scores >0.4 were considered as antigenic. Finally 15 peptides were predicted as epitopes of B-cell response (Table 29).

<table>
<thead>
<tr>
<th>Position</th>
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<tbody>
<tr>
<td>24</td>
<td>NRPAGAIPSPYKDGSSNNSE</td>
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<tr>
<td>99</td>
<td>GQCNSFYIPRHVKKEESFQ</td>
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<td>136</td>
<td>ECPGLDPFPRLKKIQKVQC</td>
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<td>72</td>
<td>WCKTQPLRQTVSEEGCRSRT</td>
<td>0.901</td>
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Table 29: Predicted epitopes of B-cell response for testis specific antigen 62
Genetic, molecular and microbiological basis for ovarian carcinoma from a low incidence population

S.Shanmughapriya, Department of Biochemistry, Bharathidasan University

.................EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
DRQKVLTLERCSKLEGELHKRTEIIRSLTKKVALESNQMECQTALQKTLQLQEMAQK 120
EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
ATHSSLSEDLEARNETSNTLVELLHORQAREQALTTMIKLKDIIEAVNHIADEC 180
EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
SGKFMLEHALDADAKMAETCIVKEKQDKQKLKALIEVNLKDLNEKTTENNQREEI 240
.................EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
IRLQEKSLHDELLFTVERERKRKDELLNIAKSKQERTNSLHNLRLQYVQKQSDLQFLN 300
EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
FNVQNSQELIQMYDSKMEESKDALSSRMDCLSDLKNNHLPVDIKREKNQKSLFKDQKFAE 360
........................................EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
MLVQQRSDKSSCDCEKEKQDITVFGEKSVITLSSLIFTKDLVKEKHLNFLWGLGKTQIE 420
........................................EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
PENKITLCKIHTKSPKCHTGQVEQKQPSETPTLSDEKQWHDSVYLGTLNCPSKHE 480
........................................EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
KLVECQDQMERSIEISSCCQNACQLGESMCDSKCCHPSNFIIHEAPGHMDSVEWMSIFK 540
........................................EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
SKMRQIVLKSCTCSEICGTQHDSPASELIAIQDSHSLGSSKSLREDETESSNKKN 600
........................................EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
SPTSLLIYKDAPNEAESIVLPSQDFSPSTKLQRSLLAESRQMVTDELSLPLISHEN 660
........................................EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
LTGSATKSEVESSQAKTNFVSY 684
........................................EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE

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<td>EKTTENNEQREEIIRLQEK</td>
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<td>551</td>
<td>SGCTCSEICGTQHDSPASE</td>
<td>0.878</td>
</tr>
<tr>
<td>16</td>
<td>SEVEISTIEKQKELQLLIG</td>
<td>0.87</td>
</tr>
<tr>
<td>257</td>
<td>TVERERKRKDELLNIAKSKQ</td>
<td>0.837</td>
</tr>
<tr>
<td>414</td>
<td>GGKTQIEPENKITLCKIHTK</td>
<td>0.824</td>
</tr>
<tr>
<td>302</td>
<td>NVENSOQELIQMYDSKMEESK</td>
<td>0.801</td>
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<tr>
<td>127</td>
<td>LSEDLEARNETSNTLVELS</td>
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</tr>
<tr>
<td>483</td>
<td>DVECQDQMERSIEISSCCQKNE</td>
<td>0.776</td>
</tr>
<tr>
<td>363</td>
<td>VQQNRSKDSSCDECEKKQQ</td>
<td>0.761</td>
</tr>
<tr>
<td>76</td>
<td>EGELHKRTEIIRSLTKKVKA</td>
<td>0.741</td>
</tr>
</tbody>
</table>

6.3.4.3 C16orf88
C16orf88 is also called as testis specific antigen 118. The testis specific antigen 118 belongs to the coiled coil repeat family. The ontology of the protein is unknown. The subcellular localization of the protein is nucleolus.

The sequence of the testis specific antigen 118 analysed by VaxiJen v2.0 server gave a score of 0.7368 showing the protein as antigenic in nature. The BCPreds predicted the epitopes for B-cell response. The epitopes with VaxiJen scores >0.4 were considered as antigenic. Finally 14 peptides were predicted as epitopes of B-cell response (Table 30).

**Table 30: Predicted epitopes of B-cell response for C16orf88**

<table>
<thead>
<tr>
<th>Position</th>
<th>Epitope</th>
<th>Score</th>
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</thead>
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<tr>
<td>62</td>
<td>PLVKKKKKKKKGVSTLCEEH</td>
<td>1</td>
</tr>
<tr>
<td>279</td>
<td>PVIEEPALKRRKKKRKESG</td>
<td>1</td>
</tr>
<tr>
<td>256</td>
<td>IPISDDPKASAKKKMKSKKK</td>
<td>1</td>
</tr>
<tr>
<td>146</td>
<td>KKLKKHKKEKKGQADPTAFS</td>
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<tr>
<td>13</td>
<td>PEKKKKKKKVKEPETRSVL</td>
<td>1</td>
</tr>
<tr>
<td>235</td>
<td>MESSPRKGSKKPKVKEAPE</td>
<td>1</td>
</tr>
<tr>
<td>213</td>
<td>KKKKIQEGLPGHSKPS</td>
<td>0.999</td>
</tr>
<tr>
<td>125</td>
<td>HASGVKTSQDERGEEETRV</td>
<td>0.997</td>
</tr>
<tr>
<td>301</td>
<td>GDPWKEETDTDLGVLKEKKG</td>
<td>0.98</td>
</tr>
<tr>
<td>83</td>
<td>EPEETLPARRTEKSPLRKQ</td>
<td>0.956</td>
</tr>
</tbody>
</table>

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Cancer-testis antigens are a group of tumor antigens, expressed in normal testis and different types of tumors. As their name implies, their expression is seen in germ cells of the testis but sometimes they are expressed in female reproductive organs and trophoblasts (Kalejs and Erenpuis, 2005; Zendman et al., 2003; Simpson et al., 2005). Immature germ cells of fetal ovary (oogonia and primary oocytes) express cancer-testis antigens but their expression has not been seen in oocytes in the resting primordial follicles. Cytotrophoblast and syncytiotrophoblast of the placenta express some cancer-testis antigens (Old, 2001). Expression of these antigens in the placenta is different from other antigens; it means that some of them are not expressed in the placenta but some are highly expressed, and their expression is not completely paralleled with their presence in the fetal germ cells (Jungblunth et al., 2007).

Because some characteristics of malignant tissues, such as invasiveness, destructiveness, and metastatic features, are shared with trophoblastic cells, gene expression profile in the placenta can be similar to cancer. Some cancer-testis antigens can be expressed in nongametogenic tissues such as the pancreas, liver, and spleen at levels much less than germ cells (Scanlan et al., 2004). In addition, it was recently reported that some cancer-testis antigens such as N-RAGE, NY-ESO, MAGE, and SSX are expressed in both adult and fetal human mesenchymal stem cells of the bone marrow but after differentiation of osteocytes and adipocytes, their expression is down-regulated (Cronwright et al., 2005). It has been suggested that expression of cancer-testis antigen in addition to be a special characteristic of gametogenesis can be a stem cell marker. This restricted expression of these antigens in undifferentiated somatic and germ cells is suggestive of their essential role in embryonic development (Costa et al., 2007). Cancer-testis antigens are considered as promising target molecules for cancer vaccines because of their highly tissue restricted expression (Simpson et al., 2005; Scanlan et al., 2004; Parmigiani et al., 2006; Meklat et al., 2007).

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
<th>Score</th>
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<tbody>
<tr>
<td>331</td>
<td>RRKALQEEIDRESGKTEASE</td>
<td>0.936</td>
</tr>
<tr>
<td>34</td>
<td>NDDYFADVSPRATPSKSV</td>
<td>0.905</td>
</tr>
<tr>
<td>405</td>
<td>GKKAAADSLQQNLQRDYDRAM</td>
<td>0.857</td>
</tr>
<tr>
<td>435</td>
<td>GFSTAPNKIFYIDRNASKSV</td>
<td>0.804</td>
</tr>
</tbody>
</table>
Until now at least 70 families of cancer-testis gene with 140 members have been attributed to this group and their expression has been studied in different types of tumors (Stevenson et al., 2007). Some of them are proved to be immunogenic. Attributing genes to this gene group is based on some characteristics: 1) mRNA expression in normal tissues is almost limited to testis, fetal ovary, and placenta, 2) mRNA expression in different cancers.

In the present study two cancer testis antigens (testis specific antigen 62 and C16orf88) have been found to have strong immunogenic properties. The sequence analysis of these proteins by VaxiJen score also substantiated the antigens to be highly immunogenic. Thus these two cancer-testis antigens can be considered as promising target molecules for cancer vaccines in this population because of their highly tissue restricted expression.

6.3.4.4 Ceruloplasmin

Ceruloplasmin belongs to multicopper oxidase family and it contains three F5/8 type A domains and 6 plastocyanin like domain. The major function of this protein is ferroxidase activity oxidising Fe$^{2+}$ to Fe$^{3+}$ without releasing oxygen species. The protein is involved in ion transport across cell membrane. Thus the major biological function of the protein is ion process/ cellular iron ion homeostasis and transmembrane transport. The important molecular function of the protein was considered to be chaperone binding. The subcellular localization of the protein is secreted in the plasma.

The sequence of ceruloplamin protein analysed by VaxiJen v2.0 server gave a score of 0.4966 showing the protein as non-antigenic in nature. The BCPreds predicted the epitopes for B-cell response. The epitopes with VaxiJen scores >0.4 were considered as antigenic. None of the epitopes were predicted to be antigenic.

In this regard, ceruloplasmin cannot be utilized for the development of vaccine and alternatively it can be hypothesised the utilization of the ceruloplasmin promoter to develop a cancer specific promoter for the purpose of targeted gene therapy. To develop a cancer-specific promoter (CSP), it is important to determine the genes that are highly expressed in cancer because some of the genes that are preferentially
expressed in cancer are transcriptionally activated. That is, the overexpression of genes in cancer may be due to strong promoter activity. If overexpression is due to transcriptional up-regulation, the promoters of the overexpressed genes would be more active in cancer than in normal cells, and therefore can be used to drive a therapeutic gene to target cancer cells for effective gene therapy and minimizing side effects. Based on the relative expression ratios (ovarian cancer versus normal) available in the literature, several promoters that are more active in ovarian cancer cells (Tanyi et al., 2002; Tzukuman et al., 2000; Bao et al., 2002) have been identified. Among these, the ceruloplasmin promoter is more specific to ovarian cancer cells.

The ceruloplasmin serves as a cofactor in various physiological enzymatic reactions including a role in copper transport (Luza et al., 1996), maintenance of vessel tone (Cappeli-Bigazzi et al., 1997a,b; Bianchini et al., 1999), and antioxidant properties, which has implications in disorders including Parkinson’s and Alzheimer’s diseases (Patel et al., 2002). High levels of ceruloplasmin expression have been demonstrated in various cancers such as thyroid carcinoma (Kondi-Pafiti, 2000) and melanoma (Cox et al., 2001). Dysregulation of copper transport due to ceruloplasmin expression in tumors has been studied by suppressing copper with tetrathiomolybdate in head and neck tumors in clinical trials (Cox et al., 2001).

It was originally identified and cloned with evidence that the regulatory CAAT/enhancer-binding protein elements were not responsible for tissue specificity (Bingle et al., 1993). Mutations in the ceruloplasmin promoter appear not to be responsible for the dysfunction in iron modulation in patients with diseases such as hemochromatosis (Lee et al., 2001). The ceruloplasmin promoter appears to be regulated by hypoxia-responsive elements and hypoxia-inducible factor-1 (Mukhopahyay et al., 2000) as well as iron deficiency anemia. Because the ceruloplasmin promoter is regulated by hypoxia and anemia, it may serve as a useful tool in gene therapy.
Protein 1 – 62.1 kDa Testis specific antigen 62

Protein 2 – 19.3 kDa protein Grelin-2

Figure 43: Spectrum of the proteins identified by MALDI-TOF analysis

1 MAVSEIKPKLKNLTKVPISHKRNIDLPQGLCQLQHQKEKKLSASMQA
51 AFQDANYFNYKTDQCGMDHGMCTVAKLQMNLDVYNELKCAIDDRD
101 GKNDFDKVLDNFLKAVPKETCDLALGPGILLFELRLLET
151 SALPRKSIIEISVYPQRFQHTGPGMLWSPYTMGKRTLKDCTPSS
201 SMAAFANAAARIAIMEKLDFKLEELKRCNPSGSDPSYKIFLPNVD
251 GVVMKPKFKMDQIKLKEITHERYFFHHKRDAKTOAANIKSMDPAS
301 GYSNFTIVDPQLKKKCTTVDATAIKQHVRATTDYNGLIAHLREKEM
351 LNLWQIRGDGILGMDRSNFQYDTFTSTYTW5WVNCQELLSPKDLRLYDAY
401 VNRNSSHSR5SSSSDSCYTDSSGRKRDNGKLGDFQOQ

1 MFWKLSSLF LVAVLVKVEAARKNRPGAIKPSYKMDGSSNNSERWQHIIK
51 EVLASSQALKVTERKLSDWKCTQPLQVTSEEGCRSERILRNCYGQ
101 CNSYIPRHVKKKEEESQSCAFCPQVRITSLVELECPGLDPFPRLKQQ
151 KVKQRCMVSNLSDSDKQ

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Protein 3 – 51.5 kDa C16orf88

1 MITKTHKVDL GLPEKKKKKK VVKEPETRYS VLNNDDYFAD VSPLRATPS
51 KSVAHGQAPE MPLVKKKKK KKGVSTLCEE HVPEPETTLPA RRTKEPSLRR
101 KQVFGHLEFL SGEKKNKKSP LAMHASSGVK TSPDPROQGE ETRVGGKLKK
151 HKKKEKKGAQD PTAESVQDPW FCEAREARFD GDTCSVGKKD EEQAALGQXR
201 KRKSPREHNG KVKKKKKKHQ EGDALPGHSK PSREMSSPR KGSKKPKVVK
251 EAPEYIPISO DPKASAKKKM KSKKVEQPV IEPEAKRKK KKKKRESGVA
301 GDPWKEETDT DLEVLEKKKG NMDEAHHIDQV RRKALQEEID RESGKTEASE
351 TRKWGTQFG QWDTAGFENE DQKLKFLRLM GGFKNSPSF SRPASTIARP

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Protein 4 – 150 kDa Ceruloplasmin

1 MKILILGIFL FCSTPAWAK EKHYIYIGKE TTWDYASDHG EKKLISVDTE
2 HSNNLYQNGP DRIGRLYKKA LLYQYTDTEF RRTIIEKPVVW GFLGPIIKAE
101 TGDKVVHULK NLASRPYTFH SQHITYYKEH EGAIFPDNTT DFOARDDKVY
151 PGEQYTMIL ATEEQSPGEQ DGNCVTRIYH SHIDAPKDIAS SGILGPLIC
201 KDSDLKEKE KHDREFVVM FSVVDenFSW YLEDNITYC SEPEKVDKDN
251 EDFQESNRMV SVQNYFTGSL PGLSMAEDR VKWLFGMGN EVDVHAFFH
301 GQALTNKNYR IDTINLFPAT LFDAYMAQNP PGEWMLSCQN LNHKAGLQA
351 FFQVQECNKS SSXDNIRGKH VRHYHIAAE IEWNYAPSGI DIFTKENLTA
401 PGSQAVFFE QGTTRIGGSY KKLVYREYTD ASFTNRKREG PEEEHLGLG
451 PVIWAENVGDT IRVTFHNGA YPLSIEIPGV RFNKNNEGY TSPNYPQPSR
501 SVPSSASHVA PTEFTYEWET VPKVEGPTNA DPVCMLAKMY SAVDPTDKIF
551 TGLIGPMKIC KGSSLHANGR QKVDKEFYL FPVTFDENES LLEDDNIRMF
601 TTAPDQVDKE DEDFQESNKM HSMNGFMYGN QPLTMCKGD SVVWYLFSAG
651 NEADVHGYF SGNYTLWRGE RRDTANLPQ TSSTLHMPD TEGTFNECL
701 TTDHYTGGMK QKYTVNQCRR QSESTSFYLG ERTYIAAEVE VEWDYSQPQRE
751 WEKELHLHLE QNVNSAFLDK GEFYIGSKYK KVYIQYRTDS TFRVPERKA
801 EEEHLGILGP QLHADVGDKV KIFKMNAR PYSHAHVGQ TESSTVPTPL
851 PGEFLVYVVK IPERSGAGTE DSAIPWAYV STVQVVDLQY SGLIGPLIC
901 RPRYYLFHNP RRKLEFALLV LVEFDESWSY LDDNITYSD HPEKNVDKDE
951 EFIESNKMHIA INGRMFNGLQ GLTMHVGDDEV NWYLMGGMN EIDLHTVHFG
1001 HSFQXKHRYV YSSVDFDIFP GTYQTEMFIP RTPGIWLHHC HVDTHIHMAG
1051 ETYTIVLQNE DTKSQ

S. Shanmugapriya, Department of Biochemistry, Bharathidasan University
6.4 Summary and Conclusion

The present study identified 4 immunodominant protein namely gremlin-2, C16orf88, testis specific antigen-62 and ceruloplasmin. Out of the four proteins identified, two were testis specific protein which is not expressed or expressed at very low concentration in somatic cells on comparison with germ cells. Three proteins were found to be immunodominant and predicted to be antigenic by their VaxiJen score in the range of 0.6135 to 0.7368. Thus the three proteins (gremlin-2, C16orf88, testis specific antigen-62) can be utilised for the development of epitope based vaccines. The protein ceruloplasmin is specifically expressed in ovarian cancer cells. Ceruloplasmin promoter is regulated by hypoxia and anemia, a condition that prevails in ovarian cancer cells and thus the ceruloplasmin promoter can serve as a useful tool in gene therapy. Thus all the four proteins identified can be utilised for clinical applications (Figure 45).

*Figure 45: The clinical applications of the proteins identified by MALDI-TOF analysis*
SUMMARY & CONCLUSION
7.0 Summary and Conclusion

- The significant risk factors for the development of ovarian carcinoma in this study population was found to be full time employment which increased the risk 3 times on comparison with non-employed women, early age at menarche which increased the risk 6 times on comparison with women with late menarche and post menopausal status which increased the risk 3 times on comparison with pre and peri menopausal women.

- The study showed the ovarian carcinoma of the study population to be hormone dependant with consistent association between ovulatory events or ovulation associated with ovarian inflammation.

- Of all the ovarian carcinoma cases analysed, 18 (25%) of the cases were found to contain one or more of $p53$ variations.

- One of the $p53$ variations was found to be polymorphism thus leaving 17 alterations detected in ovarian carcinoma.

- Nine of the $p53$ mutations were found to be frameshift, 7 deletions and 2 were insertions.

- All the $p53$ mutations reported in the present study were confined to the DNA binding domain with deleterious functional defects including loss of transactivation of RGC, BAX, PIG3, PCNA, MDM2, Waf1.

- As a result of mutations at codon 139 and 138, there is a upregulation of MRP1 and CDDP resistance, which contribute to the drug resistance of the ovarian carcinoma patients and in failure of the first line chemotherapy.

- No significant differences were found in $p53$ mutation in regard to histological types of tumour.

- The correlation between $p53$ mutation and history of narcotics use showed that the narcotics users are at a reduced risk of acquiring $p53$ mutation compared with women without a history of narcotics use.

- The study on the $I655V$ polymorphism in HER2 gene showed that 27.7% of the case patients and 40.3% of the control subjects were heterozygous for
Valine allele and 69.4% of the case patients and 10.7% of the control subjects were homozygous for this allele.

Compared with Ile/Ile genotype women with Val/Val or Val/Ile genotype of the HER2 gene had an elevated risk of ovarian cancer.

The genotype distributions in this study population were consistent with the Hardy-Weinberg equilibrium with Valine allele frequency as 0.30 which is similar to that of the German Caucasian subjects.

There was no statistically significant difference between the groups of patients with different genotypes (AA, AG, GG) regarding the histological types.

A joint effect of HER2 polymorphism and factors related to endogenous estrogen exposure such as age at menarche and late menopause has been demonstrated.

The immunohistochemistry analysis showed HER2 protein to be strongly expressed in 38.5% of the patients.

The relationship between HER2 polymorphism and protein overexpression showed a statistically significant association.

In total 8 (11.1%) of the 72 ovarian carcinoma patients were found to be positive for BRCA mutation.

None of the cases were found to be positive for founder mutations, 5382insC in BRCA1 and 6174delT in BRCA2.

The mutation frequency for BRCA1 was found to be 6.9% and for BRCA2 it was 4.2%.

One mutation 1750delA has been identified in three BRCA1 carriers thus this mutation alone contribute to a mutation frequency of 4.2%. Thus this particular mutation can be considered as a founder mutation in case of the study population.

Mean age at diagnosis of ovarian cancer patients with BRCA1 mutation was 55.6 and for BRCA2 it was 36. BRCA2 carriers were found to be significantly younger when compared to BRCA1 carriers.
BRCA1 mutated cases were found to be of serous adenocarcinoma and BRCA2 carriers as serous cystadenoma.

From the results of the mutation studies it can be concluded that I655V polymorphism plays an important and major role in the development of ovarian carcinoma among this study population followed by p53 mutation and BRCA. Thus the neoplasm is hormone dependent, and “ovulation” mechanism determine the level of risk. If the clinical history of a suspected women favours hormone dependant risk then they can be screened for HER2 polymorphism and overexpression. The treatment regimen in a such can be the use of Herceptin (anti-HER2 antibody) coupled with chemotherapy drugs that enhances the survival rate of the patients. The screening program for mutations can include the aforementioned mutations in the exons of BRCA1, BRCA2 and p53.

The other aetiologies for ovarian carcinoma are

1. Nearly 21 actinomycetes isolates have been obtained from the endocervical swabs of the women categorized into five groups.
2. In the present study out of the 9 ovarian carcinoma cases who had a history of IUD usage, 4 were found to be positive for actinomycetes infection.
3. Of these four cases, 3 were reported clinically to have previous history of PID.
Through this we propose a hypothesis of simulation of ovarian carcinoma by actinomycetes species that have colonized the IUD.

The use of IUDs facilitates the colonization of actinomycetes which in turn leads to PID and pelvic actinomycosis. Further pelvic actinomycosis simulates pelvic malignancies.

Out of the 21 actinomycetes isolates, three showed (A4, C15, C17) showed high biofilm forming ability.

These three isolates were characterized as *Nocardia* sp. strain C15, *Nocardia* sp. strain C17 and *Streptomyces* sp. strain A4.

*Nocardia* sp. strain C17 showed high biofilm forming ability on the copper sheets resisting copper ions.

The biofilm formed by these isolates were found to be susceptible to the polyene antibiotic nystatin at a concentration of 0.16mg/ml.

The probable mechanism of biofilm inhibition by nystatin is by the inhibition of the twitching motility of the isolates.

38.5% of the ovarian carcinoma cases were found to have HPV infection.

The results showed HPV 6 to be the probable infecting HPV genotype.

The positive amplification for the E6 and E& genes of HPV showed the integration of the HPV genome into the host genome resulting in consequence of neoplastic transformation of ovarian epithelium.

At baseline 80% of the ovarian carcinoma cases were found to be positive for *Chlamydia* infection.

Approximately 50% of the cases showed detectable CMV DNA.

Thus in conclusion if the case patients have a previous history of PID or IUD usage then they can be screened for actinomycetes infection followed by treatment with linezolid and gentamycin antibiotics. As a prophylactic measure nystatin coated IUDs can be commercialized. Since 80% of the ovarian carcinoma cases are affected by *Chlamydia* infection, the use of antibiotics including amoxicillin, azithromycin, doxycyclin, erythromycin, tetracycline and ofloxacin can be used to augment chemotherapy regimen.