ANALYSIS OF HLA-A2 FREQUENCY AS PROGNOSTIC AND/OR RISK FACTORS IN OVARIAN CANCER PATIENTS

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To my family

CONTENTS

1 ABSTRACT

Major histocompatibility complex antigens are mandatory for the immune response, and genetic imbalance may be linked to tumour escape. The HLA-A2 allele in Scandinavia has a high prevalence; it decreases with latitude and also with ovarian cancer mortality in Europe. In solid tumours, HLA-A2 has been suggested to be a risk factor and a negative prognostic factor.

Study I. 32 unrelated Swedish women with relapsing or progressive ovarian cancer were analyzed for the genotypes at the HLA-A, -B, -Cw, and -DRB1 loci by PCR/sequence-specific oligonucleotide hybridization. The frequencies of HLA alleles of healthy Swedish bone marrow donors provided by the coordinating centre of the Bone Marrow Donors Worldwide Registries, Leiden, The Netherlands were used as controls. The HLA-A1 and HLA-A2 gene and phenotype frequencies were increased, while HLA-A3 was decreased in ovarian cancer patients compared to healthy Swedish donors. HLA-A2 homozygotes were 2-fold higher in patients. HLA-B15 and HLA-Cw3 were only present in HLA-A2 positive patients hence the HLA-A2-Cw3 and -B15 haplotypes were segregated. Furthermore, in these patients, A2-B5, A2-B8 A2-DRb1*03 and A2-Drb1*03 haplotypes were also increased, but not segregated.

Study II. In order to confirm the assumption, that HLA-A2 may have a role in the prognosis of ovarian cancer, we have examined the presence of HLA-A2 in all patients with ovarian cancer, admitted to the department of Oncology-Pathology, Karolinska Institute, over a 1-year period (1995) and related its presence to survival. A population-based set of 97 patients with confirmed epithelial ovarian cancer were recorded in a database by age, histology, stage, surgery and treatment. At the time the study was initiated, the majority of the patients were not alive and HLA-A2 expression was therefore determined by PCR/sequence-specific oligonucleotide hybridization using DNA extracted from paraffin-imbedded tissue specimens. 88 patients with a median age of 65 years (36–87) could be evaluated. 44% were serous adenocarcinomas, 28% endometrioid, 6% mucinous, 13% clear cell carcinomas, 7% undifferentiated and 2% other epithelial tumours. Stages I–II comprised 33% and stages III–IV 67%. In stages III–IV and serous histology, 73% were HLA-A2 positive. Cox analysis, in this group, showed high univariate (HR7.16; CI 2.04–25.03; P = 0.002) and multivariate (HR 6.8; CI 2.10–22.4; P = 0.001) Hazard Ratios. None of the HLA-A2 positive patients survived 5 years, compared to more than 50% of the HLA-A2 negative patients.

Conclusion. In cohort of 32 patients with relapsing or progressive ovarian cancer, there are indications of an unusual overrepresentation of HLA-class I-II genes/haplotypes, as well as segregation for the HLA-A2-Cw3 and -B15 haplotypes. Investigation on a larger series of ovarian cancer patients confirmed that HLA-A2 is a negative factor for survival in women with serous adenocarcinomas of the ovary in stages III-IV. This finding has implications for clinical patient management. Association with known oncogenes needs further analysis.

2 LIST OF PUBLICATIONS

The thesis is based on the following original articles, which are referred to in the text by their Roman numerals:

I. Gamzatova Z, Villabona L, Van der Zanden H, Haasnoot G. W., Andersson E, Kiessling R, Seliger B, Kanter L, Dalianis T, Bergfeldt K, Masucci G. V. High expression of HLA-A2 and HLA-A1 gene frequency and HLA-A2-B15-Cw3 haplotype segregation in a cohort of patients with advanced stage ovarian cancer.
Submitted under 2nd revision in "Tissue Antigens".

II. Gamzatova Z, Villabona L, Dahlgren L, Dalianis T, Nillson B, Bergfeldt K, Masucci G. Human Leucocyte Antigen (HLA) A2 is a negative clinical prognostic factor in patients with advanced ovarian cancer. Gyn. Oncology, 2006, 103 (1): 145-150.

3. LIST OF ABBREVIATIONS

Adc-Adenocarcinoma

beta2m-beta2microglobulin

BRCA1, BRCA2-Breast Cancer genes

C2, C4 и B (Bf)-components of complement

CIN-Cervical Intraepithelial neoplasia

CTL (CD8+)-Cytotoxic T Lymphocyte

EGF-Epidermal Growth Factor

EOC-Epithelial Ovarian Cancer

FIGO-International Federation of Gynaecology and Obstetrics

G-Grade of tumour differentiation

H-W test-Hardy-Weinberg disequilibrium test

HER-2/neu- Human Epidermal growth factor Receptor

HLA-Human Leukocyte Antigen

HPV-Human Papilloma Virus

HSD-Healthy Swedish bone marrow Donors

HSP-Heat shock protein

IFN-alpha-Interferon-alpha

Ig-Immunoglobulin

mAb-Monoclonal Antibody

LT-Lymphotoxin

MHC-Major Histocompatibility Complex

NK-Natural killer

PBMCs-Peripheral Blood Mononuclear Cells

SDS-sodium dodecyl sulphate

SSO(P)-Sequence Specific Oligonucleotide (Probes)

SSP-Sequence Specific Primers

TAP-Transporter Associated with antigen Processing

TCR-T Cell Receptor

Th (CD4+)-T helper

TNF-Tumour Necrosis Factor

TP53- Tumour Protein

VIN-Vulval Intraepithelial Neoplasia

4 BACKGROUND

4.1 Epithelial ovarian cancer (EOC)

Surface epithelial – stromal tumours are the most common neoplasms of the ovary. They originate from the ovarian surface epithelium or its derivatives and occur in women of reproductive age and beyond (Tavassoli F. A., Devilee P., 2003).

4.1.1 Epidemiology

Ovarian cancer accounts for approximately 4% of all malignancies in women, worldwide, although there are major geographical disparities in incidence and mortality (Bray F. et al., 2005). In 2002 more than 204,000 women in the world were diagnosed with ovarian cancer and around 125,000 women died from the disease. Incidence rates are highest in developed countries, with rates in these areas exceeding 9 per 100,000. Incidence in Asia and Africa is less than 7,2 cases per 100,000 (Parkin M. D. et al., 2005). Cancer of the ovary represents about 30% of all cancers of the female genital organs. (Tavassoli F. A., Devilee P., 2003). Ovarian cancer is the fourth most frequent cause of cancer death in women and accounts for 5% of all cancer deaths. The death rate from ovarian cancer exceeds that of cervical and endometrial carcinomas combined. (Hoskins W. J. et al., 2005). Age-standardized mortality rate for ovarian cancer varies from 7.9 in Northern Europe to 4.5 cases per 100,000 in Southern Europe (Parkin M. D. et al., 2005).

4.1.2 Aetiology

The molecular events leading to the development of epithelial ovarian cancer are unknown. Epidemiologic studies have identified endocrine, environmental and genetic factors as being important in the carcinogenesis of ovarian cancer. Epidemiologically established risk factors include nulliparity, family history, early menarche and late menopause (Daly M., Obrams G. I., 1998).

Genetic factors

Many (at least 60) cellular oncogenes have been identified; however, their involvement in ovarian cancer has yet to be fully elucidated (Yang G. et al., 2004; Mammas I. N. et al., 2005; Inan S. et al., 2006; Kolasa I. K. et al., 2006; Psyrri A. et al., 2005). Epidemiologic studies and detailed analysis of familial ovarian cancer pedigrees have consistently confirmed the existence of two distinct manifestations of hereditary ovarian cancer. The first is the breast-ovarian cancer syndrome, in which these cancers are seen in excess, sometimes in the same individual. This has been linked to the BRCA1 gene at chromosome 17q12-21 and, to a lesser extent, to the BRCA2 gene at chromosome 13q. The second manifestation is ovarian cancers associated with an excess of colorectal and endometrial cancers that define the

hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, also known as Lynch syndrome II (Lynch H. T. et al., 1998; Farrell C. et al., 2006; Lee J. S. et al., 2006; Sogaard M. et al., 2006).

Reproductive factors

Epidemiological studies have demonstrated that parity and oral contraceptives using are important protective factors in ovarian cancer (Pollock R. E. et al., 2004; Pelucchi C. et al., 2007; Yang C.Y. et al., 2007). The increased risk of ovarian cancer in women with a prior history of breast cancer as well as the two-to fourfold increased risk of breast cancer in women with a history of ovarian cancer provide further evidence of the importance of altered hormonal environment in the etiology of ovarian cancer. Close medical surveillance, and perhaps even prophylactic oophorectomy, might be justified in these high-risk groups. (Risch H. A., 1989, Bergfeldt K. et al., 2002). Cancer Prevention Study II, a large prospective study conducted by the American Cancer Society, recently reported an association between ovarian cancer mortality and postmenopausal estrogen use (Rodriguez C. et al., 2001). Elevated androgen levels have been associated with an increased risk of ovarian cancer, while progestins may, on the other hand, be protective (Risch H. A., 1998; Shildkraut J. M., 2002). Recently published data provide evidence that endometriosis is associated with increased risk of ovarian cancer (Prowse A. H. et al., 2006; Kobayashi H. et al., 2007).

Environmental factors

Total dietary fat, saturated fat consumption, serum cholesterol levels, dairy foods, lactose intake and the use of talc-containing cosmetic products have been reported to be associated with ovarian cancer risk in small case-control studies (Chang S., Risch H. A., 1997; Zhang M. et al., 2002; Fairfield K. M. et al., 2001; Mills P. K. et al., 2004; Larsson S. C. et al., 2006). However, no statistically significant relations were found for these factors and increased risk of epithelial ovarian cancer (Huncharek M. C. et al., 2003; Kuokkanen M. et al., 2005; Koralek D. O. et al., 2006 and Mommers M. et al., 2006).

4.1.3 Presentation

The most common presenting symptom is that of abdominal discomfort or pain, followed closely by abdominal distention due to the presence of malignant ascites or large intraabdominal masses. Gastrointestinal symptoms are also relatively frequent, and the symptoms of nausea, dyspepsia, early satiety, constipation and/or obstipation are common but, unfortunately, nonspecific. Occasionally, patients will experience urinary frequency or disuria and/or vaginal bleeding (Hoskins W. J., 2005). An elevated ovarian cancer antigen assay (CA-125) will predict cancer in over 80% of postmenopausal women with a palpable adnexal mass. The chest x-ray may

reveal pleural effusion. An abdominal ultrasound or CT scan may determine the presence of hepatic metastases (Weiss G. R., 1993).

4.1.4 Diagnosis and staging

Epithelial ovarian cancer is classified according to the cell type (serous, mucinous, endometrioid, clear cell, transitional cell, mixed epithelial, undifferentiated and unclassified adenocarcinomas), grade (well-, moderately- or poorly differentiated), and stage (I, II, II, IV) (Benedet J. L. et al., FIGO Committee on Gynecologic Oncology). Ovarian cancer is staged surgically. There should be histologic confirmation of the disease. Operative findings, prior to tumour debulking, determine stage, which may be modified by histopathologic as well as clinical or radiological evaluation (Heintz A. P. M. et al., 2003).

4.1.5 Screening

Screening for ovarian cancer is not recommended for the general population, because currently available screening tests do not achieve high levels of sensitivity and specificity. The advantage of screening is much higher for women at high risk (such as those with a strong family history of ovarian cancer and those with BRCA1 or BRCA2 mutations). Serial transvaginal sonography, with or without Doppler imaging and CA-125 measurement, is recommended for them (Hensley M. L., 2000; Menon U., Jakob I. J., 2000; Fields M. M., 2006).

4.1.6 Treatment

First-line treatment for ovarian cancer currently relies on aggressive cytoreductive surgery combined with chemotherapy (Gadducci A. et al., 2001; Hoskins W. J. et al., 2005). Cytoreduction, combined with chemotherapy, may relieve symptoms associated with bowel obstruction and improve survival (Ozols R. F., 2002). The optimal surgical procedure for all epithelial ovarian carcinomas is complete abdominal exploration, removal of the uterus along with fallopian tubes and ovaries, omentectomy, lymph node sampling, random peritoneal biopsies, including diaphragm, aspiration of ascites or peritoneal lavage (Hoskins W. J., 2005). The two-drug combination of carboplatin plus paclitaxel is the current standard regimen for advanced ovarian cancer (Ozols R. F., 2006**).** Although chemotherapy approaches featuring taxanes and platinums, when given following optimal cytoreductive surgery, can increase the survival of patients, treatment of metastatic disease eventually results in drug resistance and disseminated disease can not be cured. Therefore, novel treatment approaches are needed. For epithelial ovarian cancer and many other solid tumours, a multimodality approach using the combination of cytoreductive surgery, chemotherapy, gene therapy and immunotherapy might be needed. Gene therapy in ovarian cancer includes several strategies, such as replacement of an altered tumour suppressor genes, inhibition of growth factor receptors; molecular

chemotherapy, antiangiogenic gene therapy and virotherapy. Immunotherapy of ovarian carcinoma consists of tumour vaccines, adoptive therapies with antitumour T cells, therapies targeting regulatory T cells and cytokine therapy.

4.2 MHC and cancer

Human tumour cells in a number of malignancies, including EOC, are recognized as nonself by the immune system and elicit an immune response. Ovarian tumours are infiltrated by tumour-infiltrating lymphocytes (TILs), which are mostly composed of T cells, and they may represent an immune response of the host to the tumour (Platsoucas C.D. et al, 2003). Tumour-infiltrating lymphocytes are present in both malignant ascites and solid tumours from patients with EOC (Freedman R.S., Tomasovic B. et al., 1994; Freedman R. S., Edwards C. et al., 1994). The ability of T lymphocytes to recognize peptide epitopes, derived from cytoplasmic and nuclear proteins, when these are presented in association with self-MHC class I molecules at the surface of an antigen-presenting cell (Townsend A. R. M. et al., 1985), potentially allows the immune system to respond to the changes in the transformed cell, and places HLA molecules at the center of the immune response to tumours (Browning M., Dunnion D., 1997). The relationship between HLA and immunological recognition of tumour cells has been pushed to the forefront once more through an increased understanding of the process involved in both tumour development and tumour immunity (Browning M., Dunnion D., 1997).

Despite the key role of MHC molecules for anti-tumour immune responses, the number of studies analysing associations between HLA expression and cancer incidence as well as prognosis, are still limited. There are a series of reports suggesting associations between HLA-A2 antigen expression and increased risk of cancer development and prognosis. HLA-A2 was negative prognostic factor in non-small cell lung carcinoma (So T. et al., 2001). Trend for shorter survival was observed in HLA-A2 positive patients with chronic myelogenous leukemia (Cortes J. et al., 1998). Overrepresentation of HLA-A2 allele was observed in vulval intraepithelial neoplasia patients (Davidson E. J. et al., 2003). HLA-A2 conferred the highest risk of squamous cell carcinoma of the cervix (Montoya L. et al., 1998). HLA-A2 was a risk factor for breast cancer as well (Biswal B. M. et al., 1998). HLA-A2 was higher in nasopharyngeal carcinoma patients than in controls (Lu C-C. et al., 2003).

4.3 MHC and ovarian cancer

De Petris L. et al., 2004 observed an overrepresentation of HLA-A2 phenotype in patient's group compared to the healthy Swedish population ($p = 0.01$). As it is already known, the higher phenotype frequency of this allele found in Scandinavian countries decreases significantly, as one moves further south in Europe. Ovarian cancer mortality rates decrease, as well as the demographic changes in HLA-A2. These observations have to be confirmed

by more extended investigations in order to elucidate if HLA-A2 higher frequency is already present at the diagnosis (risk factor) or is selected during the course of the disease (prognostic factor). Moreover, this fact would suggest different strategies for specific immunotherapy in addition to first line conventional treatments. In order to further investigate possible associations between ovarian cancer and HLA-A2 expression in ovarian cancer we expanded the study performed by De Petris L. et al., including additional patients, and performed a complete HLA typing in 32 and HLA-A2 typing in 88 ovarian cancer patients.

4.4 The major histocompatibility complex (MHC)

The human major histocompatibility complex (MHC) region encompasses over 4 Mb of DNA (~0.1% of the genome) on the short arm of chromosome 6 at **6p21.31** (Campbell R. D., Trowsdale J., 1993, 1997). The MHC is traditionally divided into the class I, class II and class III (Lechler R., Warrens A., 2000). Many genes have been identified within the genetic boundaries of the MHC, encoding complement components, cytokines and other molecules with a role in immune responses. These other genes are sometimes referred to as MHC class III genes (Lechler R., Warrens A., 2000).

4.4.1 Structure of MHC class I and class II molecules

MHC class I and class II genes, while evolutionarily related, and generally very similar to each other, yet have subtle differences in both structure and function. The class I genes code for the α polypeptide chain of the class I molecule; the β chain of the class I molecule is encoded by a gene on chromosome 15, the β_2-microglobulin gene. There are some 20 class I genes in the HLA region; three of these, HLA-A, -B and -C, the so-called classic, or class Ia genes. The class II genes (HLA-DP, -DQ, -DR) code for the α and β polypeptide chains of the class II molecules. The immunoglobulin-like domains of class I and class II molecules contain the binding sites for the T cell co-receptors CD8 and CD4, respectively (Klein J., Sato A., 2000; Abbas A. K., Lichtman A. H., 2003).

4.4.2 The class I region

The class I region contains 2 Mb of DNA. It contains three main functional class I loci, HLA-A, HLA-B, HLA-C, all of which are highly polymorphic (Lawlor D. A. et al., 1990). Most somatic tissues at varying levels express these genes. The non-classical class I loci include HLA-E, HLA-F and HLA-G (Geraghty D. E., 1993). These genes, which are generally much less polymorphic than HLA-A, -B or -C lead to the production of class I-related molecules of restricted tissue distribution. HLA-E is involved in instructing NK cell receptors (Ulbrecht M. et al., 1992; Braud V. M. et al., 1997, 1998). HLA-G is expressed on fetal trophoblast cells and is thought to play a role in

the maternal tolerance of the fetus (Loke Y. W., King A., 1991; Parham P., 1996). HLA-F gene has been implicated in the development of hereditary haemochromotosis (Feder J. N. et al., 1996).

4.4.3 The class II region

The class II region spans over 800 Kb of DNA and contains one gene every 40 Kb on average. These include all of the known class II α- and β-chain genes. In humans HLA-DP, -DQ and -DR are expressed mainly on the surfaces of specialized antigen-presenting cells (APCs), such as dendritic cells, macrophages, B-lymphocytes and a few other cell types, including endothelial cells and thymic epithelial cells (Lechler R., Warrens A., 2000; Abbas A. K., Lichtman A. H., 2003).

4.4.4 Function of MHC class I and class II molecules

The major function of the MHC molecules is to assist the display of unique molecular fragments of antigens on the surface of cells in the arrangement that permits their recognition by immune effectors such as T-lymphocytes (Naik S., 2003). The antigen receptors of T cells are unable to recognize antigen directly; they can only see foreign antigens in the form of short segments of peptide bound to MHC molecules. The MHC role in the presentation of antigen was explained by the MHC- restricted recognition of antigens (Zinkernagel R. M., Doherty P. C., 1975). MHC molecules bind only one peptide at a time. Peptide binding is of low affinity, and the off-rate is very slow, so that complexes, once formed, persist for a sufficiently long time to be recognized by T cells. The peptide-binding cleft of class I molecules can accommodate peptides that are 8 to 11 amino acid residues long, whereas the cleft of class II molecules allows larger peptides (up to 30 amino acids residues in length or more) to bind. The polymorphic residues of MHC molecules are localized to the peptide-binding domain. Some polymorphic MHC residues determine the binding specificities for peptides by forming structures, called pockets that interact with complementary residues of the bound peptide, called anchor residues. CD4+ helper T lymphocytes recognize antigens in association with class II MHC gene products, and CD8+ CTLs recognize antigens in association with class I gene products (Abbas A. K., Lichtman A. H., 2003). The function of the MHC in immune responsiveness is also reflected in its genetic polymorphism (the presence of multiple allelic variants of a gene). HLA genes are the most polymorphic genes in man. The evolutionary pressure over the centuries has resulted in generation of this polymorphism, so that we are equipped to face the multitude of infectious challenges we face (Naik S., 2003).

5 AIMS OF THE STUDY

- To investigate HLA genes distribution in ovarian cancer patients compared to the healthy population;
- To identify HLA haplotypes distribution in ovarian cancer patients compared to the healthy population;
- To find risk and/or prognostic relevance of HLA-A2 gene in ovarian cancer patients.

6 MATERIAL AND METHODS

Subjects (I and II)

The studies were performed at the Karolinska University Hospital, in Solna, the Cancer Centrum Karolinska and in Huddinge, Immunologic laboratory.

Group I was comprised of 32 Unrelated Swedish women with advanced or relapsing epithelial ovarian cancer, heavily treated with more than two lines of chemotherapy. The diagnosis of ovarian carcinoma in all 32 cases was histologically proved. These patients were recruited for the HLA-A2 restricted peptide vaccination trial at the Gynecologic oncology unit.

Group II included 147 patients with the preliminary diagnosis of ovarian cancer, chronologically admitted to the Gynecologic oncology unit during the year 1995. The primary objective of II study was to find the prognostic relevance between HLA-A2 and well-defined clinical parameters. Re-examination of histological diagnosis excluded 50 patients due to other diagnosis, such as borderline ovarian tumour, non-epithelial ovarian carcinoma, cancer of genital tract (except ovaries), cancer of extragenital localization and relapse of ovarian cancer. These 50 cases were not further analyzed.

Group I and II: The median age in a group I was 51.5 years (range 31-80 years), in a group II - 65 years (36-87). The diagnosis of epithelial ovarian cancer was made according to WHO histological classification of tumours of the ovary. Staging was based on surgical and pathological findings, according to the International Federation of Gynaecology and Obstetrics (FIGO) classification of the tumours of the ovary. Clinical evaluation and imaging studies had been done as appropriate **(Benedet J. L. et al., FIGO Committee on Gynecologic Oncology).** Material for the HLA typing was extracted from peripheral lymphocytes and paraffin-embedded tissue blocks of the patients with histologically proved diagnosis of epithelial ovarian cancer. Written informed consent was obtained before study entry. Consent form, procedures and protocols, used in this study, were reviewed and approved by the Karolinska University Hospital Ethical Committee (#KI-99-318# and #KI-03-619#). The specimens used for all the analysis (**I and II**) were coded.

Methods (I and II)

DNA extraction protocol.

Group I: Blood samples were drawn from 32 patients in 10 ml citrate treated tubes for HLA-A, -B, -C and HLA-DRB1 testing. Peripheral lymphocytes were isolated from collected blood samples by addition of Lymphoprep according to manufacturer's protocol (Axis-Shield PoC AS, Oslo, Norway). Genomic DNA was extracted from isolated lymphocytes using "Roche High-

Pure DNA extraction kit procedures 030625" (Roche, Molecular Biochemicals, Manheim, Germany) according to the manufacturer's protocol. Samples of 200 µl were treated with 1 ml extraction buffer, mixed, incubated for 30 min at 80°C and then centrifuged for 10 min at 12 000 g. Supernatant was collected into a new reaction tube with 400 µl binding buffer and 80 µl proteinase K. This mixture was further incubated for 10 min in 72°C adding 200 µl isopropanol and then loaded onto a filter tube placed on a collection tube and centrifuged for 1 min at 5000 g. These samples were washed twice with 450 wash buffer at 5000 g centrifugation for 2 min. After the final wash step, samples were dried by a 10 min centrifugation at max speed. DNA in the filter was then eluted with 50 µl elution buffer into a new reaction tube by a 1 min centrifugation at 5000 g. Finally the DNA amount and purity were measured by NanoDrop technology.

Group II: DNA was extracted according to the protocol for "Roche High-Pure DNA extraction kit procedures 030625" (Molecular Biochemicals, Mannheim, Germany). The paraffin was removed by xylene and ethanol. The tissue pellet was disrupted by overnight treatment at 55°C in tissue lysis buffer, SDS and proteinase K. On day two, the DNA was bound to the membrane in collection tubes, washed and then eluted. Proteinase K treatment was repeated (1 hour, 55°C) for removal of trace proteins. The DNA was bound to the membrane in a fresh collection tube, washed and eluted. DNA amount and purity were measured by NanoDrop technology.

HLA-PCR (I and II)

Group I: HLA genotyping was performed using the Olerup SSP HLA Typing Kit (Olerup SSP AB, Stockholm, Sweden). PCR products were separated by electrophoresis at 150 V for 30 min on a 3 % agarose gel, stained with ethidium bromide, and visualized under UV light. Results were evaluated then for HLA gene sequences.

Group II: Approximately 120 ng of DNA was run in a PCR with primers specific for the HLA-A2 gene (provided by Olle Olerup, SSP AB, Saltsjöbaden, Sweden). The samples from HLA-A2 positive patients gave rise to a PCR amplicon of 124 bp, while samples from HLA-A2 negative patients gave no PCR amplicon. The 20 µl PCR mixture contained 120 ng sample DNA, 2.0 pmol of each primer and 6 µl Master Mix. The Master Mix contained 2 U of Taq polymerase, 200 µM of each dNTP, 50 mMKCl, 1.5mMMgCl2, 10 mMTris–HCl with pH 8.3, 0.001% w/v gelatine, 5% glycerol and 100 µg/ml cresol red. Amplification was run in an automated thermocycler (GeneAmp PCR system 9700, Applied Biosystems). The cycles consisted of an initial denaturation of 2 min at 94°C followed by 10 cycles of 94°C for 10 s and 65°C for 60 s, then 20 cycles of 94°C for 10 s, 61°C for 50 s and finally 72°C for 30 s. Water was used as a negative control, and DNA extracted from HLA-A2 positive patients was used as a positive control. PCR products were run on a 3.0% agarose gel stained with ethidium bromide and visualized under UV light. To avoid false negative, a PCR was run with

primers directed to the S14- and β2-globulin gene, which are present in all human cells. S14 PCR was preformed as previously described (Mellin H, et al., 2002). Water was used as a negative, and DNA extracted from human fibroblasts as a positive control. PCR products were run on a 3.0% agarose gel stained with ethidium bromide and visualized under UV light.

Control group. The control group comprised 40162 healthy Swedish transplant donors for the HLA loci A, B, and DRB1 and 350 for the HLA locus Cw, respectively. These data were provided by the coordinating centre of the Bone Marrow Donors Worldwide Registries, Leiden, The Netherlands.

Statistics (I and II)

Group I: The evaluation of the data presented in the paper is based on a cohort of patients already in the progress of the disease and treatments, (considered as a prevalent sample) of the Stockholm County ovarian cancer population). The administration of these patients in the Stockholm county (2 million inhabitants) is mainly (95%) referred to Karolinska Oncological clinic. The data presented are therefore descriptive and part of the follow up project that will determine the final validity of the findings. HLA class I and II genotype frequency was calculated by counting the number of alleles present for each HLA type divided by the total number of alleles. Haplotype frequency (HF) was calculated using a manual method (Schipper R. F. et al., 1997; Schipper R. F. et al., 1998). In brief, heterozygous and homozygous patients for HLA-A2 haplotypes were counted as 1 event. Only in the case of complete haplotype homozygosity were they counted as 2 events. The sum of the events (A) was then divided by the total number of alleles (B) and reduced to percentage (haplotype frequency %) = (A/B) x 100). The statistical significance of the association of individual HLA alleles or haplotypes with the cohort of patients described here was calculated using the Fisher's exact test. Two-tailed p-values were calculated to detect positive and negative associations. Results with a p-value of 0,05 are expected to be significant. The p values have been corrected to reduce the possibility of false positive results (Bonferroni correction). Corresponding p-values were obtained using chi-square2 test (Svejgaard A., Ryder L. P., 1994). Additionally, since this is a retrospective analysis it is more proper to use the Odd Ratio (OR) according to Wollf-Haldane method (Woolf B., 1955; Haldane J.B., 1956). It was determined to define the power of association of EOC to HLA alleles. The OR is the ratio of the odds of the risk factor in the EOC and in the Healthy Swedish Donors (HSD) obtained by the cross product of the entries in a 2x2 table. The OR >1 indicates a positive and a <1 a negative association, whereas =1 defines no disease association. Due to the small sample size available in this analysis Confidence interval (C.I.) at 95% for OR has also been calculated in order to avoid a rejection of the null hypothesis based only on the 2x2 table p values and hence to see how large the difference between the proportion in EOC patients and HSD. For comparison of the gene expression we have also simply considered the data obtained by the registry of

HSD expressed as a ratio by dividing the observed percentage of frequency in patients by the percentage observed in the donors. For the A2-Cw3 and B15-Cw3 haplotypes we have applied the Hardy-Weinberg disequilibrium test in order to determined the degree of segregation/disequilibrium (Delta) according to the formula: $D = (d/N)1/2 – [((b+d)/N) ((c+d)/N)]1/2$.

Group II: The $\chi 2$ trend test was used to examine patient characteristics for discrete categorical variables or factors. Overall survival was the end point for the study, with a statistical event defined as death from any cause. Survival time was calculated using date of first diagnosis and date last seen or date of death. HLA-A2 positive patient was regarded as 1 and negative - as 0. Cumulative survival plots and time-to-event curves were constructed using the Kaplan-Meier product-limit method, with the log-rank test applied to detect differences between groups. Diagnostic data were collected during the year 1995 and were censored at the 31st of October 2003. Univariate Cox regression analyses were performed for each prognostic factor. These factors were age, clinical stage, histologic type and HLA-A2 phenotype. Hazard Ratios (HR) and 95% confidence intervals (CI) were estimated. To test the assumption of proportional hazards, an interaction term of a prognostic variable and a time-dependent covariate were added. A significant effect of that interaction term denotes the presence of a time dependent effect and thus a violation of the proportional hazards assumption. Then, multivariate Cox regression analyses were performed including binary coding of all factors with a stepwise procedure. P values < 0.05 were considered statistically significant. All analyses were performed with the program StatView for Windows, SAS Institute Inc. Version 5.0.1.

7 RESULTS

In paper I we analyzed a cohort of EOC patients for the complete HLA genotype including the HLA-A, -B, -Cw, -DRb1 alleles and the frequencies of the distinct haplotypes together with survival. This group of patients was characterized by an overrepresentation of stage III and IV tumours compared to the patient population at diagnosis and the national FIGO distribution. Two patients were diagnosed at stage I (6.25 %) and 2-at stage II (6.25 %). Seventy five percent were stage III and 12.5% were stage IV. Compared to the histopathology, recorded at diagnosis, 75% of the patients in this cohort had serous adenocarcinomas, 12.5% had endometrioid ovarian cancer, 3.1%-mucinous adenocarcinoma and 9.4%-presented other histological types (clear cell, mixed epithelial and undifferentiated carcinomas).
The majority (87.5%) of the patients received primary surgery, but in only 19 % it was radical. Twenty two percent of patients obtained a second surgery and all the patients in this cohort received platinum-based treatment. Fifty nine percent received more than 6 chemotherapy cycles and 22 % were also treated with external radiotherapy.

Analysis of HLA

According to the recruitment criteria all patients were typed for the HLA class I locus, HLA-A, -B, -C genes as well as the HLA class II locus HLA-DRb1. For patients #18 and # 21 no complete HLA typing was available. The frequency of the most common genotypes and phenotypes was compared to the healthy Swedish population. Considering the genotype, EOC patients had a higher ratio both in genotype and phenotype for the HLA allele A1 (1.21) and A2 (1.31), but a lower frequency in the HLA-A3 allele (0,65). Among the B locus the genotype frequencies of HLA-B8 (1.42), and HLA-B15 (1.27) had the highest ratios. The class II allele HLA-DRB1*03 exhibited approximately a 2-fold increased frequency when compared to the healthy Swedish donors (1.9). The OR CI and Fisher 2-tailed test was performed for the phenotype frequency between the two groups. Since the EOC group is limited in number there are no significant corrected p values. The OR gives the opportunity to consider the increase of HLA-A1 (1.5; C.I. 0.74-3.10) and HLA-A2 (1.6; C.I. 0,75-3.25) and the HLA-A3 decrease (0.6; C.I. 0.29-0.15). HLA-B8 (1.99; C.I. 0.96-4.10) and DRB1*03 alleles had the highest OR. Remarkably, both HLA-B15 and HLA-Cw3 were only recorded among the patients expressing HLA-A2. In addition, a higher HLA-A2 homozygote frequency (22 %) was detected in the patients compared to the expected frequency in healthy Swedish donors (12 %) (p=0,05, corrected p=0.08). However, no homozygotes in the HLA-A1 and -A3 alleles as well as other common alleles were found. Sixteen out of 22 (73%) HLA-A2 positive

patients had a serous adenocarcinoma and the majority, but 2, in clinical FIGO stage III-IV.

Haplotypes analysis

Patients' haplotype frequencies were compared to those of the HSD by OR, C.I. and Fischer's $\chi 2$ p and corrected p value. All haplotypes analysed had OR above 1. HLA-A2-B5 and A2-B8 had a strong OR and a C.I. above 1. The p value was significant for HLA-A2-B8, the corrected p was also significant. HLA-A2-B15, HLA-A2-DRB1*03, HLA-A2-DRB1*04, HLA-A2-B15-Cw3 and HLA-A2-B8-DRB1*03 had high OR, C.I. above 1 and significant p, but not after Bonferroni's correction. HLA-A2-Cw3 and HLA-B15-Cw3 was increase in the frequency is mostly due to the segregation, compared to the HSD. In spite of the limited number of EOC cases, the Hardy-Weinberg's HF was for HLA-A2-Cw3 1309.04 (delta 338.08) compared to the HSD (H-W HF 773; Delta 64) which is significant (p =0.04) and H-W HF for the HLA-B15-Cw3 was 600.96 (delta 0.03) compared to HSD (H-W HF 237; Delta -3,88).

In paper I we detected an unusual overrepresentation of the HLA-A2 antigen as well as segregation for the HLA-A2-Cw3 and -B15 haplotype in selected cohort of patients with advanced ovarian cancer. In order to verify our assumption that HLA-A2 may have a role in the risk and/or prognosis of ovarian cancer, in paper II we have examined the presence of HLA-A2 in all patients with ovarian cancer, admitted to the Gynaecologic Oncology unit over the 1-year period (1995) and related its presence to survival.

The paraffin-embedded tissues, from 97 patients, were collected for possible detection of HLA-A2 by PCR. Material from nine patients could not be analyzed due to fragmented DNA. The remaining 88 patients were tested and suitable for further analysis. The distribution of histological type was 44% seropapillary, 28% endometrioid, 13% clear cells, 6% mucinous, 7% undifferentiated and 2% unclassified. The most frequent clinical stages were IIIc (36%), IV (25%) and Ic (15%). Stages I–II (33%) and stages III–IV (67%) were arbitrarily merged. Age, stage, grade of differentiation and histology distribution of the population that were evaluated do not differ from the figures released by the FIGO annual report for European countries (Pecorelli S. et al., 1998). HLA-A2 was detected in 57% of the patients by PCR. Age, either $\leq$50 or >50 years, did not affect the distribution of HLA-A2. Presence of HLA-A2 gene in the patient's genotype was subsequently matched to the histological type of the tumour. An overrepresentation of HLA-A2 positive patients was observed among patients with serous adenocarcinoma. More specifically, 26/39 (67%) patients with serous adenocarcinoma were HLA-A2 positive and 24/33 (73%) HLA-A2 positive patients had stages III–IV tumours. In contrast, HLA-A2 was equally distributed among the group with endometrioid ovarian cancer as well as among G1, G2 and G3 grades of differentiation. Distinction by clinical stage

resulted in a higher proportion of HLA-A2 positive patients with tumour stages III and IV, compared to that of patients with stages I and II tumours. This was more evident in merged stages. In clinical stages I–II, the percentage of HLA-A2 positivity was less than in the total population, although the difference was not significant. Fifty-nine percent of patients with stages III–IV expressed HLA-A2. Women with stages III–IV and serous histological type were mostly HLA-A2 positive (73%).

Ninety-four percent of the patients were primarily treated surgically. Chemotherapy, most commonly platinum-based (88%), was the first line of treatment in 94% of the cases. The treatment modalities, applied to HLA-A2 positive and HLA-A2 negative patients, did not differ ($\chi 2 = 2.07$, $p = 0.72$).

Univariate test was used to analyze the impact of well-known factors such as age, clinical stage, histology, grade, as well as the presence of HLA-A2. Age was included in the analysis as continuous time factor, significantly relevant for survival with a Hazard Ratio (HR) 1.41. Survival did not differ between groups of patients aged either 50 years or younger vs. those above 50 years of age. Serous adenocarcinomas did not affect the death rate more than other histological types of tumours. Grade of differentiation (HR = 2.54) seemed also to be a prognostic factor. HLA-A2 positivity in the non-stratified patient population had an HR=1.45. This was a tendency towards a higher risk for death, on the other hand, p=0.13, which implied a possibility to make a wrong assumption from the H(0) hypothesis. Stages I–II were indifferent to the presence or absence of HLA-A2. On the contrary, stages III–IV patients with HLA-A2 had significantly higher HR 2.15, compared to HLA-A2 negative patients (CI 1.19–3.87 and p=0.01). Stages III–IV and serous adenocarcinoma histology, grouped together, registered the highest numbers of patients expressing HLA-A2 as well as a 7.16 times higher risk to die than the HLA-A2 negative patients (CI 2.04–25.03; P = 0.002). The proportional Hazard Ratio was calculated also by multivariate analysis considering the total population age, merged clinical stages, grade of differentiation, serous histology and presence of HLA-A2. Taken together, stages III–IV (4.53; CI 2.10–35; $p < 0.001$) and HLA-A2 phenotype expression had a significantly high Hazard Ratio (1.71;CI 1–2.86; p=0.04). On the other hand, grade 3 vs. grade 1-2 and serous histology vs. all other histology types were not affecting survival. These findings were strongly emphasized by stratification either for stages III–IV, for grade or for serous histology. In the latter, presence of HLA-A2 increased the HR to 6.8 (CI 2.10–22.4; p=0.001). Stratification by stage showed, as expected, that the higher clinical stages III–IV have a significantly higher mortality rate than the lower stages. In stages III–IV, the presence of HLA-A2 predicted an 80% death rate the first 2 years from diagnosis compared to that in HLA-A2 negative group (50% cumulative survival). Grade 3 patients as well as stages III–IV, HLA-A2 positive patients had low survival rate compared to HLA-A2 negative patients. This relationship was much stronger if serous adenocarcinoma was considered in the stratification. None of the HLA-A2 positive patients with clinical stages

III–IV and serous tumours survived longer than 5 years after diagnosis, while in the same group, more than 50% of HLA-A2 negative patients were still alive after 5 years from the diagnosis.

8 DISCUSSION

In addition to the control of transplant acceptance and immune responsiveness, it has been recognized for many years that the MHC in the humans plays an important role in the aetiology of a number of diseases. The highly frequent association of some diseases with a specific HLA allele is clinically useful in the diagnosis of these conditions (Naik S., 2003).

Despite this established knowledge, the number of studies looking at associations between HLA-expression and incidence as well as prognosis in different types of cancer are sparse, and for ovarian cancer even more than sparse. However, there are number of papers suggesting associations between expression of HLA-A2 and an increased risk of developing some types of cancer, although this association seems to be most established in virus related malignancies, such as vulva cancer in situ (Davidson E. J. et al., 2003), cervical cancer (Montoya L. et al., 1998), and nasopharyngeal cancer (C-C. Lu et al., 2003). HLA-A2 was negative prognostic factor in non-small cell lung carcinoma (So T. et al., 2001). Trend for shorter survival was observed in HLA-A2 positive patients with chronic myelogenous leukemia (Cortes J. et al., 1998). HLA-A2 was a risk factor for breast cancer as well (Biswal B. M. et al., 1998).

Paper I: We have previously described an increased frequency of the HLA-A2 allele among metastatic ovarian patients and observed that the mortality rates for ovarian cancer correlate with HLA-A2 gene frequency in European countries (De Petris L. et al., 2004). In the present study, we have extended the investigation in this cohort by analysing the expression of HLA-A, -B, -Cw and -DRb1 loci as well as the incidence of HLA-A2 homozygotes and HLA-A2 associated haplotypes.

The majority of the patients had serous adenocarcinomas and were predominantly stage III-IV; the latter was a result of the recruitment criteria of this cohort. Patients with other stages of the disease relapse less frequently after primary therapy, and therefore were not available for the cohort. The HLA-A2 phenotype was more frequent in the patients analysed, compared to healthy donors. Additionally, occurrence of HLA-A2 homozygotes was almost 2-fold higher, compared to the Swedish donors, and almost 3-fold higher, compared to the median European population. These data demonstrate that, in the cohort of patients described here the degree of malignancy in combination with the HLA-A2 phenotype identifies a group of patients, out of the population at diagnosis, who require immediate and continuous oncological care due to frequent relapse and short survival.

Furthermore, a lower presentation of patients with the HLA-A3 phenotype was found with a difference of more than 30 % when compared to that of the donors. In several reports the role of HLA-A3 and its efficiency in

antigen presentation has been discussed (Bocchia M. et al., 1995). HLA-A3 has also been correlated with poor prognosis in autoimmune diseases (Clark R. E. et al., 2001; Fogdell-Hahn A. et al., 2000) as well as having a protective feature in malignancy (Posthuma E. F. et al., 1999). This leads to the hypothesis that HLA-A2 is not a good prognostic factor for monitoring specific immune responses against tumours (negative prognostic factor). On the other hand, this could be corroborated by the diminished presentation of HLA-A3 among these patients (possible positive prognostic factor). However, this assumption has to be confirmed by more extensive analysis in therapy naïve patients observed during a long period of time.

Parallel to the HLA-A2 and HLA-A1 overrepresentation, an increase in the frequency of HLA-B8, HLA-B15, HLA-DRB1*03 and HLA-DRB1*04 was observed. HLA-DRB1*0301-DQA1*0501-DQB1*0201 haplotype have been described to be associated with ovarian cancer (Kubler K. et al., 2006). Unfortunately in this paper the patients were compared with, selected by infertility and in a treatment program, control group. In our paper we confirmed the findings using a selected EOC cohort of patients already in treatment in our clinic. In addition, our results showed increased HLA-A2-B8 and HLA-A2-B8-DRb1*03 haplotypes. These findings suggest a wider haplotype association to advanced EOC than that described by Kubler K. et al., 2006.

In contrast, the HLA-Cw3 and -Cw7 phenotype and genotype frequencies were not numerically overrepresented when compared to the healthy Swedish population, but all the HLA-Cw3-positive patients as well as HLA-B15 were segregated to the patients expressing HLA-A2. The segregation was also extended to the haplotype HLA-A2-B15-Cw3. Furthermore, the HLA-DRb1*04 was also highly represented among this particular cohort. The combination HLA-A2-B15-Cw3-DRb1*04 has been described in several reports as one of the ancestral haplotypes which is associated with familiar conditions such as Behcet's syndrome (Fallingborg J. et al., 1986), Felthy syndrome (Runge L. A. et al., 1986) as well as familial diabetes type 1 (Cudworth A. G., 1979). The association and segregation with advanced stages of ovarian cancer is intriguing. A larger number of therapy-naive patients as well as of donors should be analyzed in order to determine whether the ancestral haplotype might be linked to high risk of acquiring this type of cancer.

The MHC complex plays a central role within the immunological integrity of the ovary due to the dynamic activity of immune cells (macrophages and T lymphocytes) presented in the ovary tissue (Best C. L. et al., 1996). Several investigations have studied the role of MHC in the interaction between immunocompetent cells and the tumour escape mechanism (Zhang L. et al., 2003; Norell H. et al., 2006) . The segregation of HLA-A2 and HLA-Cw3 is interesting and introduces a potential relationship between malignancy and prognosis through immunological escape mechanisms. The HLA-Cw3 and -Cw4 have been associated with the

recognition of the Killer cell Immunoglobulin-like Receptors (KIR) (Wagtmann N. et al., 1995; Colonna M., Samaridis J., 1995). The HLA molecule/peptide complex not only acts as a ligand for the T cell receptor, but also for natural killer (NK) cell receptors, suggesting an important role for both adaptive and innate immunity (Uhrberg M., 2005).

All the HLA-Cw, HLA-B4 and some other HLA molecules are involved in the killing and apoptosis, inducing activity of T cells as well as NK cells through the binding to the KIRs (Snyder G. A. et al., 1999). Since the KIR genes are located on chromosome 19, their hereditary transmission is independent of the HLA system. The activity of T and NK cells is regulated by the interaction of the HLA class I peptide complexes on the surface of the target cells with their activating and/or inhibiting KIR (Le Bouteiller P. et al., 2002). The impact of the KIR/HLA interaction in malignant diseases was demonstrated for hematopoietic cancers, solid tumours as well as for non-malignant diseases such as viral infections after stem cell transplantation.

Paper II: The results, obtained in this study show that the presence of HLA-A2 in ovarian cancer patients is relevant to their outcome. This surface marker is expressed in almost all somatic cells and is genetically inherited. The percentage of patients with HLA-A2 among the total patient population, compared to that of the merged clinical stages, was not different. Stratification by histology revealed that the highest number of patients with HLA-A2 was detected among that in stages III–IV and in particular in patients with ovarian cancer with serous histology. This is in line with our original observations, which pointed out that the geographical distribution of HLA-A2 genotype in Europe correlated to mortality in ovarian cancer and the description of a cohort of patients with an exceptionally high frequency of the HLA-A2 genotype (De Petris L et al., 2004). Further analysis, in a broader number of patients in that study, defined an unusual over-representation of the HLA-A2 allele with an over-representation of stages III–IV and serous ovarian carcinomas (Bergfeldt K. et al., 2005). These observations were limited by the selectivity imposed by history of previous chemotherapy, advanced course of the disease and stage and frequent relapses to which these patients were exposed to, in order to be recruited for immunotherapy.

In the present study, patients with the clinical diagnosis of ovarian carcinoma were consecutively included at the time of diagnosis to avoid selection bias due to the course and treatment of the disease. The distribution of HLA-A2 was similar to the one observed in the healthy Swedish population. Consequently, we could exclude that presence of HLA-A2 was a risk factor for ovarian cancer in the female population.

Only stratification by clinical stage (III–IV) and histology (serous adenocarcinoma) could define a cohort of patients with a specifically high percentage of HLA-A2. Presence of HLA-A2 in this latter group was also correlated to a high risk for death (100% within 5 years) compared to that

(less than 50% in 5 years) among HLA-A2 negative patients. We thus deduce that HLA-A2 could be a negative prognostic factor for patients presenting these two characteristics at the time of diagnosis. Similar associations between the presence of HLA-A2 antigen and prognosis have been described in other cancer forms (Cortes J. et al., 1998; So T. et al., 2001).

The main question is how to explain the role that HLA-A2 plays in the history of this particular tumour. At the somatic level, our findings show that Swedish women with HLA-A2 with a diagnosis of ovarian cancer at stages III–IV and a histology of serous adenocarcinoma tumours have a 7 times higher risk to die than patients with other HLA antigens and other histological types. HLA-A2 has previously been correlated to unfavorable prognosis due to immunological and genetic mechanisms, such as induction of tolerance to tumour antigens, loss of the gene at the chromosome level or down-regulation of HLA-A2 expression at the tumour cell surface level (Maleno I., Cabrera C. M., et al., 2004; Maleno I, Lopez Nevot M. A. et al., 2004). Tumour-specific antigens, restricted by HLA-A2, might have some tendency to escape from immune surveillance. The mechanisms to escape from surveillance may be ascribed either to low or absent expression of tumour-specific antigens, which bind the HLA-A2 molecule in order to induce tumour-specific resistance. Alternatively, there may be a lack or low possibility of antigen presentation due to down-regulation or low levels of HLA-A2 molecules, as well as loss of TAP-1, which contribute to antigen processing (Kaklamanis L. et al., 1995). Considering HLA-A2 as a prognostic factor for survival in serous ovarian cancer raises the question if this is linked to other genetic factors. Serous adenocarcinomas at stages III–IV have been described as a subgroup with a high frequency of genetic abnormalities (Wang N., 2002; Tibiletti M. G. et al., 2001; Auer G. et al., 1996; Meinhold-Heerlein I. et al., 2005).

Several oncogenes have been analyzed and proposed as responsible of the degree of malignancy. Remarkably, among them, the Waf1/p21 gene is located in position p21.1 on chromosome 6 about 200 Mb centromeric from the HLA-A locus (p21.3), and this could suggest a possible connection. It has also been shown that there is a correlation between the lower survival and expression of p53 and inhibition of WAF-1/p21 in the early stage of ovarian cancer (Auer G. et al., 1996). Furthermore, Bali et al. showed that, in serous adenocarcinoma of the ovary, over-expression of cyclin D1 and combined loss of p21Waf1/Cip1 in the presence of p53 overexpression, were independent predictors of overall survival (Bali A. et al., 2004). Similarly, the combination of p21Waf1/Cip1 loss and p53 over-expression was independently predictive of a shorter progression-free interval. Over-expression of p53 and cyclin E and reduced expression of p27Kip1 and p21Waf1/Cip1 were significantly associated with increasing tumour grade. In an extensive analysis of the molecular and prognostic distinction between serous ovarian carcinomas of varying grade and malignant potential, it has been shown that WAF1/p21 is not expressed in the high degree of

malignancy, while it is highly expressed in low malignant and G1 tumours (Meinhold-Heerlein I. et al., 2005) Here, we would like to emphasize the possible relationship between HLA-A2 tumour malignancy grade and prognosis.

We show that advanced stage, serous histology and grade of differentiation together with HLA-A2 positivity influence the survival adversely. Interestingly, in multivariate analysis, malignancy grade is, per se, a negative prognostic factor, but taken together with advanced stage and serous adenocarcinomas, it does not play a discriminating factor. This can be explained by the fact that the majority of the cases are in grade 3 (86%). On the contrary, it is a significant feature when analyzed for the probability of survival. At this level of investigation, we can only speculate regarding some of the possible mechanisms of action. The immune system may efficiently eliminate tumour cells with normal or up-regulated Waf1/p21, permitting the remaining cells with down-regulated or absent WAF1/p21 to escape. This could also, of course, be concomitant to a lack or downregulation of HLA expression. Rearrangements of the DNA material, cross-over, formation of microsatellite and loss of large part of the chromosomes might be the genetic mechanisms behind our findings. The relative vicinity to the HLA locus might affect the WAF1/p21 gene indirectly, and HLA-A2 allele could, in particular, be more exposed to mutations and linkages than others.

Paper I and II: The molecular events, supporting HLA subgroups as factors for tumour development or survival in ovarian cancer can only be hypothesized. There are two models suggested: (i) at the tumour level, one can speculate that tumour antigens restricted by HLA-A2 might be more prone to escape from immune surveillance and (ii) at the genetic level, HLA genes might determine susceptibility and resistance factors for the initiation of oncogenesis as well as the development of disseminated disease (Markman M. et al.,1984). Both models emphasize the need for future studies where the association of the MHC and ovarian cancer can be more thoroughly investigated including epidemiological as well as experimental studies. The results presented in our study could be the first step in the investigation of the role of HLA-A2 in advanced ovarian cancer in relation to immunological and genetic mechanisms.

However, already at this stage, knowledge of HLA-A2 implication at a clinical level should give new indications for the management of these particular patients, since despite advances in surgical approaches and chemotherapeutic agents, the overall survival rates for women with ovarian cancer have not improved significantly. Multimodality approach using the combination of cytoreductive surgery, chemotherapy, immunotherapy and gene therapy in ovarian cancer might be needed. The case of immunotherapy for ovarian carcinoma is not so well established as for other malignancies. However, the results in ovarian cancer immunotherapy compel us to continue efforts in order to optimise immunotherapy methodologies, define and

validate patient selection criteria and set combinational therapy approaches (Coukos G. et al., 2005).

9 GENERAL CONCLUSIONS

This clinical and experimental study has shown that:

- In patients with relapsing or progressive ovarian cancer, several HLA alleles, in particular HLA-A2, have higher frequencies than the healthy Swedish population. .

- In these patients there is an overrepresentation of HLA-class I-II haplotypes, as well as segregation for the HLA-A2-Cw3 and -B15 haplotypes.

- HLA-A2 is a negative prognostic factor for survival in women with serous adenocarcinomas of the ovary in stages III-IV. This finding has implications for clinical patient management.

10 FUTURE STUDIES

I. To investigate risk and prognosis in relation to HLA -A2 and B, C, DRB1 haplotypes frequency considering clinical parameters
II. To determine the incidence of ovarian cancer in relation to the HLA types in the Swedish population.
III. To investigate the possible mechanisms behind the role of HLA-A2 in the prognosis of ovarian cancer outcome in relation to possible linkage to genetic mutation and known oncogenes.
IV. To investigate loss of the HLA locus in tumours using microsatellites as genetic markers.
V. To improve the clinical outcome of the high risk patients developing immunotherapic concepts.

11. ACKNOWLEDGEMENTS

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13. APPENDIX (PAPERS I-II)