Haptoglobin Phenotype and Epithelial Ovarian Cancer

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Abstract. *Background: Haptoglobin (H) is a glycoprotein that regulates the immune response. Serum haptoglobin levels are significantly higher in patients with advanced epithelial ovarian cancer (EOC) with poor survival. Different isoforms of haptoglobin have been found in the serum of patients with EOC. We studied the genetic susceptibility and outcome of patients with EOC correlated to H phenotypes. Materials and Methods: Analyses of the H phenotypes were performed on sera from patients stored at –70˚C. A modified method based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of sera was used, followed by western blotting. Results: Seventy-nine consecutive patients with EOC and 63 healthy women were enrolled. Their mean (±S.D.) age was 58.9±12.46 years. Overall survival was 66 months (95% confidence interval=37.7-94.2). Similar distribution of haptoglobin phenotypes was observed in EOC and in healthy women. No significant correlation was found between haptoglobin phenotype, overall survival and time-to- progression. Fewer G3 tumors were found in patients with H2-2 compared with those with H1-2 (84.2% and 90.6%, respectively, p<0.04). No significant correlation was found between H phenotype and tumor markers or number of relapses. Conclusion: Although ours is a preliminary study based on a small population with scant significant findings, we hypothesize that patients with EOC with haptoglobin 2-2, might have a better prognosis because they present fewer G3 tumors and they may present a stronger immune response than patients with 1-1 and 1-2 phenotypes. Larger studies should be performed to assess the predictive value of haptoglobin phenotype in patients with EOC.*

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Epithelial ovarian cancer (EOC) is the most lethal cancer of the female genital tract. Ovarian cancer is the fourth cause of death from cancer, accounting for 6% of all cancers among women. It affects more than 190,000 women worldwide each year, with approximately 42,000 new cases in Europe per year (1) and 22,000 cases in the USA (2). Most EOCs are sporadic, with familial or hereditary patterns accounting for 5% to 10% of all cancers (3). Because of the paucity of symptoms and their insidious onset, patients present with advanced disease in more than two-thirds of cases. As many as three in ten patients whose EOC appears to be confined to the pelvis have occult metastatic disease in the upper abdomen or the retroperitoneal lymph nodes. Early diagnosis is rare. All these characteristics result in a 5-year survival of 30%. The prognosis of EOC is largely dependent on tumor stage and histological grade at presentation. The 5-year survival rate for stage I EOC is reported to be approximately 90% (1). Previous studies have also indicated that patients with well-differentiated tumors based on histological grade tend to have a better survival rate than those with poorly-differentiated tumors (2). The current management of patients with EOC requires optimum surgical cytoreduction regardless of whether surgery is performed before or after neoadjuvant chemotherapy (4). Despite an initial favorable reaction to therapy, most patients (80%) with advanced disease experiences relapse within 5 years.

The current combined use of tumor markers and ultrasonography fail to effectively screen for EOC. Although carbohydrate antigen 125 (CA-125) is the most useful biomarker for ovarian cancer, its concentrations are elevated even in women without malignancy (ovarian cysts, endometriosis, uterine fibroids, hepatic disease, renal failure, and pancreatitis); its use as a marker for early detection is thus limited. As CA-125 can only be reliably used to evaluate therapeutic effectiveness in patients diagnosed with ovarian cancer and to detect recurrent disease during post-treatment

surveillance (5-8), other biomarkers must be found that can identify patients at high risk so as to achieve early detection of EOC and to improve survival.

Over the last few years, a number of biomarkers have been identified and investigated as possible adjuncts to CA-125 screening, with haptoglobin having been proposed as a potential serum biomarker for ovarian cancer (9). Haptoglobin is an acute-phase tetrameric protein with two α/β dimmers, capable of binding hemoglobin, and thus preventing iron losses as well as renal damage. Haptoglobin is among the most abundant glycoproteins secreted by the liver, and elevation of this molecule can be observed in infection, inflammation, and in various malignant diseases, including lung and bladder cancer, leukemia, breast cancer, malignant lymphoma, urogenital tumors, and esophageal squamous cell carcinoma (9-17). Although haptoglobin is found in the serum of all mammals, a polymorphism has been reported only in humans. β-Chains are identical in all haptoglobin types and polymorphisms arise from differences in the α-chains. The main phenotypes are haptoglobin 1-1, haptoglobin 2-2, and the heterozygous haptoglobin 2-1.

We report the results of a preliminary study to investigate whether the haptoglobin phenotypes influence the risk of EOC and the outcome of this disease.

Materials and Methods

This study was approved by the Local Ethical Committee of Reggio Emilia. Study subjects were 79 patients with EOC followed-up at the Departments of Obstetrics and Gynecology of Arcispedale Santa Maria Nuova, IRCCS of Reggio Emilia, and 63 healthy women from the blood transfusion center. All 79 patients presented a histological diagnosis of EOC. All participants were recruited after obtaining their written consent.

Haptoglobin phenotype detection. Analyses of haptoglobin phenotypes were performed on sera from patients stored at –70˚C. A modified method established by Yang and co-workers (10) based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of sera was used, followed by western blotting. Haptoglobin was separated and identified using 5%-10% gradient gels. One microliter (μl) of serum was diluted by mixing 10 μl of sample loading buffer (pH 8.8; 6% SDS, 5% glycerol, 0.06 M Tris-HCl, 0.002% bromophenol blue). The mixture was then boiled for 5 min and 5 μl of pre-treated samples and 1 μl of standards, haptoglobin type 1-1 and 2-2, diluted 1 g/l (Sigma-Aldrich, Schnelldorf, Germany) were loaded onto the gel and separated for 60 min (100 V, 0.02-0.04 A). After electrophoresis, the gel was blotted onto a polyvinyl-difluoride (PVDF) membrane (Hybond ECL; Amersham, Little Chalfont, UK) pre-treated with 100% methanol by diffusion (15 min, 12 V, 0.09 A; Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell Instrument). After blocking with 5% skim milk in Tris-buffered saline (TBS) for 1 h at room temperature, the membrane was incubated with 1:1000 diluted polyclonal rabbit anti-human haptoglobin (Sigma-Aldrich, Saint Louis, Missouri, USA) used as primary antibody. After washing the membrane three times with TBS-T (Towbin-Tween), the secondary antibody: anti-rabbit IgG (Sigma-Aldrich, Saint Louis, Missouri, USA) diluted 1:1000 in TBS-T was added. The membrane was then incubated for 60 min at room temperature. Finally, the membrane was washed three times in TBS-T. The bands were visualized with enhanced chemiluminescence (ECL) reagents (Amersham) following the manufacturer's instructions. Three bands with molecular weight of 16, 20 and 42 kDa were obtained (Figure 1, lines 1-9). All serum samples from 79 patients with EOC and 63 healthy women examined exhibited the 42-kDa band. The α–bands determined the 1-1, 1-2, and 2-2 haptoglobin phenotypes.

Statistical analysis. Allelic and genotypic distribution and Hardy-Weinberg equilibrium tests are reported in Table I. The Hardy-Weinberg equilibrium test was performed using a software available on the internet (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). All other analyses were conducted utilizing the PASW Statistics 18.0 software (SPSS, Chicago, IL, USA). Quantitative variables were compared using a two-tailed Kruskal-Wallis non-parametric test. Categorical variables were compared using the Fisher's exact test or χ^2 analysis, where appropriate.

Overall survival (OS) was considered from the time of diagnosis until time of death from tumor. Time-to-progression (TTP) was considered as the time after treatment until time of the relapse. Survival curves were generated by the Kaplan-Meier method and compared using log-rank analysis. *p*-Values <0.05 were considered statistically significant.

Results

Characteristics of study participants. The mean age of patients at surgery was 58.9±12.46 years. The median value of CA-125 at diagnosis was 324 UI/ml (range 4-14145 IU/ml). Fifty-two patients (65.8%) had papillary serous adenocarcinoma, six undifferentiated adenocarcinoma (7.6%), four mucinous adenocarcinoma (5.1%), nine endometrioid adenocarcinoma (11.4%), and eight mixed adenocarcinoma (10.1%). International Federation of Gynecology and Obstetrics (FIGO) staging was obtained for all patients. Grading for three patients has not been reported (Table II).

Residual tumors after surgery were stratified into three groups. Most patients had residual tumors <0.5 cm. Neoadjuvant chemotherapy (3 cycles) was administered to 13 patients (16.5%) (Table II). Sixty-five patients (82.3%) received adjuvant chemotherapy after surgery. Thirty-six patients presented one relapse, 29 of whom received only chemotherapy (36.7%). Out of 36 patients with one relapse, seven (8.8%) patients underwent secondary cytoreduction followed by chemotherapy; 12 patients presented a second relapse, 11 (13.9%) patients received chemotherapy only (Table II).

Overall survival (OS) and time-to-progression (TTP) of the 79 EOC patients was 66 months (95% CI=37.7-94.2) and 22 months (95% CI=17.6-26.3), respectively (Table III).

Haptoglobin phenotype and its association with clinical data. The distributions of haptoglobin genotypes in 79 patients with EOC and in 63 healthy controls did not show any significant deviation from the Hardy-Weinberg equilibrium.

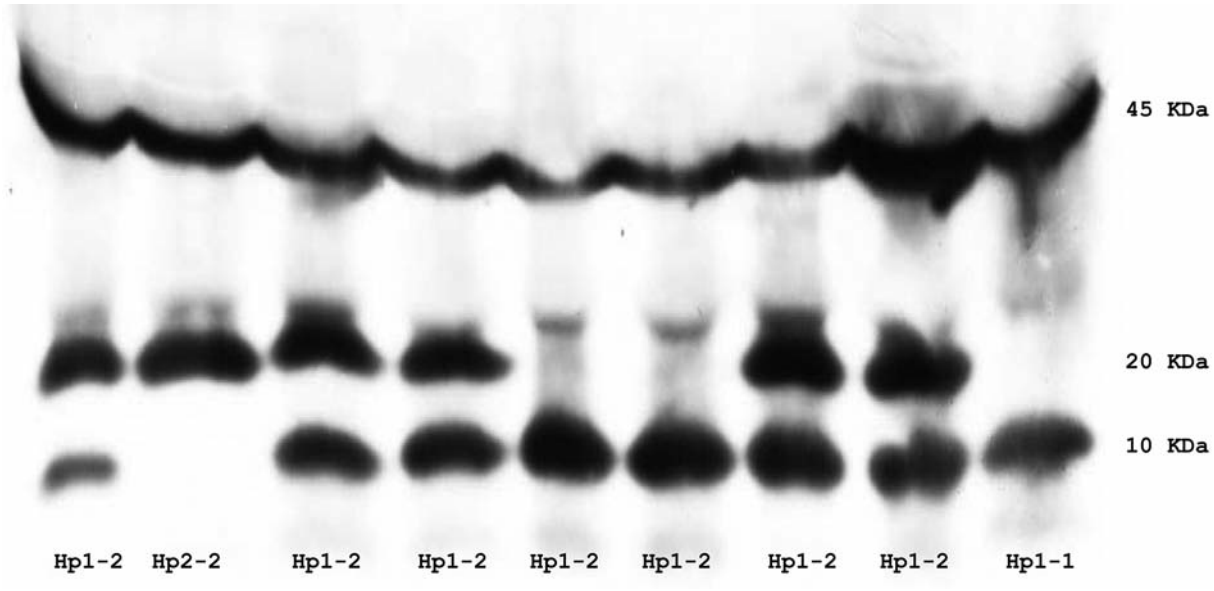

Figure 1. *Western blot analysis of serum of patients with EOC*.

Table I. *Allelic and genotipic distribution of haptoglobin in patients with EOC compared with the controls*.

	EOC patients, n=79		*p*-Value	Controls, n=63		*p*-Value
Genotypes	Observed	Expected		Observed	Expected	
Hp1/Hp1	8 (10.1%)	7.6 (9.6%)		9 (14.3%)	8.1 (12.9%)	
Hp1/Hp2	33 (41.8%)	33.8 (42.8%)	0.833	27 (42.9%)	28.9 (45.9%)	0.597
Hp2/Hp2	38 (48.1%)	37.6 (47.6%)		27 (42.9%)	26 (41.3%)	

Alleles	EOC patients, n 158	Controls, n 126
Hp1	49 (31.0%)	45 (35.7%)
Hp2	109 (69.0%)	81 (64.3%)

EOC: Epithelial ovarian cancer; Hp: haptoglobin.

Chi-square tests performed on allelic and genotypic data showed differences between cases and controls, which were not statistically significant. No significant correlation was found between haptoglobin phenotype and OS or TTP (Table III, Figure 2-3). Patients with haptoglobin 2-2 presented fewer G3 tumors compared with those with haptoglobin 1-2 (84.2% and 90.6%, respectively, $p<0.04$). No significant correlation was found between haptoglobin phenotype and tumor markers or number of relapses (data not shown).

Discussion

Since the 1970s, studies have reported increased haptoglobin levels in the serum and ascitic fluid of patients with EOC (9). Although EOC tissues may synthesize haptoglobin, this protein originates mainly from the liver. Therefore, it is reasonable to hypothesize that enhanced hepatic synthesis of haptoglobin is part of the acute-phase response to the EOC.

Studies have shown that haptoglobin levels are significantly higher in patients with advanced disease and those with poor survival (18-20), indicating that the pre-operative haptoglobin level could serve as an independent prognostic factor in patients presenting with EOC (21). Similarly to CA-125, haptoglobin also reflects response to treatment (18).

Six different isoforms of haptoglobin1 precursors were found in the serum of EOC patients; due to different glycosylation patterns, haptoglobin1 precursors are found at significantly high concentrations in early stage EOC and have thus been proposed as new biomarker for EOC diagnosis (19, 20).

Although the haptoglobin 1-1 phenotype was shown to be more frequent in patients with EOC in a previous study (22), no genetic susceptibility for EOC in our population was correlated to haptoglobin phenotype. Haptoglobin 2-2 EOC patients presented fewer G3 tumors than haptoglobin 1-2 EOC patients (84.2% and 90.6%, respectively, $p<0.04$). Although no

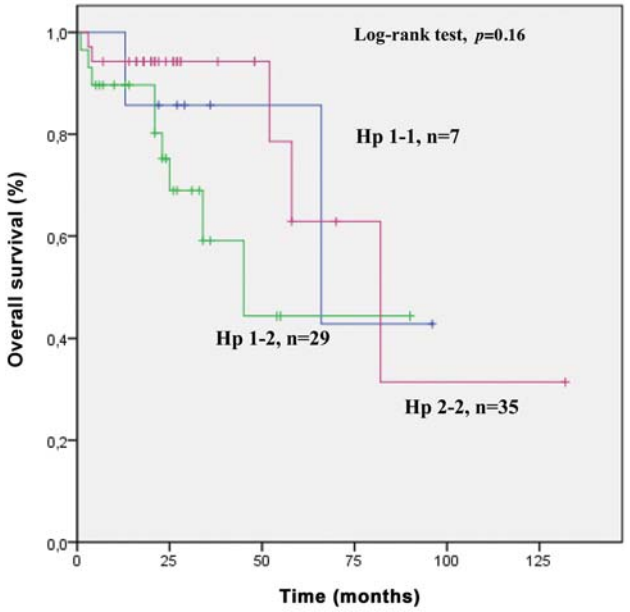

Figure 2. *Overall survival of patients with EOC according to haptoglobin phenotype: OS was 66 months (95% confidence interval (CI)=0-141.1 months) in patients with haptoglobin 1-1, 45 months (95% CI=21.7-68.2 months) in those with 1-2, and 82 months (95% CI=46.0-117.9) in those with 2-2 phenotype.*

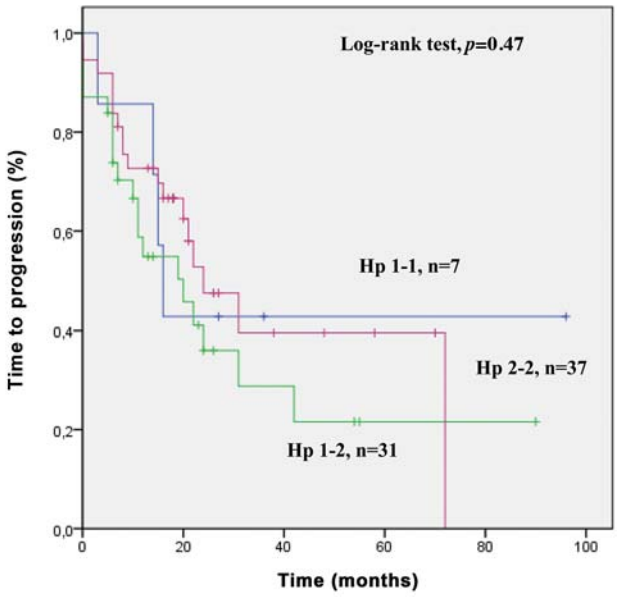

Figure 3. *Time-to-progression (TTP) of patients with EOC according to haptoglobin phenotype: TTP was 16 months (95% CI=13.4-18.5 months) in patients with 1-1, 20 months (95% CI=7.9-32.0 months) in those with 1-2, and 24 months (95% CI=13.4-34.5 months) in those with the 2-2 phenotype.*

statistical differences were found between different phenotypes and TTP, number of relapses, and OS, a clinical difference was found. Haptoglobin 2-2 phenotype in EOC, as opposed to haptoglobin 1-2 and haptoglobin 1-2, was associated with lack of recurrence (50% vs 30%), and longer TTP (16±1,3 months and 20±6.1 months for haptoglobin 1-1 and haptoglobin 1-2, respectively). These findings might indicate improved survival in patients with haptoglobin 2-2 (82±18,3 months), when compared to those with haptoglobin 1-2 and haptoglobin 1-1 (45±11,8 and 66±38.3 months respectively), which is clinically relevant. Different immune activity in association with haptoglobin phenotype may account for our findings, as anti-inflammatory and immunomodulatory activities are phenotype-dependent. Haptoglobin 1-1 phenotype directs a strong anti-inflammatory and antioxidant activity and therefore generates a TH2- dominant immune response (23-26). In contrast, the weak anti-inflammatory and antioxidant, but strong immunomodulatory activity of the haptoglobin 2-2 phenotype generates a TH1-dominant response that sustains inflammation and provides an effective immune response (24-26). Haptoglobin 2-2 is reported to be over-represented in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus and in graft *versus* host disease (27-29). A protective role of haptoglobin 2-2 was reported in patients with cervical cancer because the haptoglobin 2-2 phenotype maintains a locally active inflammatory and immune response that will inhibit HPV replication and gene expression (30, 31). Hence, the TH1-dominant response associated with the haptoglobin 2-2 phenotype might play a pivotal role in the survival of patients with EOC, who are characterized by tumor-related immunosuppressive activities. In particular, EOC typically spreads in a diffuse intra-abdominal fashion and even after recurrence, it is mostly confined to the peritoneal cavity, where it has immunosuppressive activities (32, 33). EOC presents several mechanisms to escape immune rejection such as changing antigen expression, altering the expression of the major histocompatibility complex type I, expressing immunosuppressive factors (Transforming Growth Factor β, Interleukin 10, and C-C motif chemokine 22) and by down-regulating the expression of intracellular adhesion molecules in the malignant tissue (34-39). Finally, induction of peripheral tolerance, a physiological process, is exploited by the tumor in order to evade immune elimination.

However, It has also been reported that haptoglobin is involved in immune suppression in cancer. Samak and colleagues (40) suggested that haptoglobin may act as a non-specific blocking factor protecting tumors against the host's immunological attack. Oh *et al*. (41) reported the presence of a unique epitope in a tumor-associated haptoglobin protein that manifests an immunosuppressive property in patients with ovarian, lung, and head cancer. Recently, haptoglobin was shown to alter the immune response and be involved in the pathophysiology of endometriosis and endometrial cancer, causing immune dysfunction.

Table II. *Characteristics of study participants*.

Characteristic		All patients	By phenotype			
			Hp 1-1	Hp 1-2	Hp 2-2	*p*-Value
Number		79	8	33	38	
Age, years		58.9±12.46	60.26±20.2	57.7±10.9	59.6±11.9	N.S.
FIGO	Stage I-II	20 (25.3%)	3 (37.5%)	7 (21.2%)	10 (26.3%)	N.S.
	Stage III-IV	59 (74.7%)	5 (62.5%)	26 (78.8%)	28 (73.7%)	
Grade*	G1	6 (7.8%)	2 (28.6%)	2 (6.3%)	2 (5.3%)	$p<0.04$
	G2	7 (9.1%)	2 (28.6%)	1 (3.1%)	4 (10.5%)	
	G3	64 (83.1%)	3 (42.9%)	29 (90.6%)	32 (84.2%)	
Residual tumor (cm)	<0.5	54 (74.0)	6 (75.0%)	21 (65.6%)	27 (81.8%)	N.S.
	0.6-1	11 (15.0%)	2 (25.0%)	5 (15.6%)	4 (12.1%)	
	>1	8 (11.0)	0	6 (18.8%)	2 (6.1%)	
Adjuvant chemotherapy°	Yes	65 (85.5%)	5 (62.5%)	25 (83.3%)	35 (92.1%)	N.S.
	No	11 (14.5%)	3 (37.5%)	5 (16.7%)	3 (7.9%)	
One relapse[$]	Yes	36 (46.2%)	4 (50.0%)	16 (50.0%)	16 (42.1%)	N.S.
	No	42 (53.8%)	4 (50.0%)	16 (50.0%)	22 (57.9%)	

EOC: Epithelial ovarian cancer; Hp: haptoglobin; N.S.: not significan; *statistically significant; °missing data in 3 cases; [$]in 1 case.

Table III. *Overall survival (OS) and time to progression (TTP) compared by log-rank test*.

	All patients	By phenotype			
		Hp 1-1	Hp 1-2	Hp 2-2	*p*-Value*
OS, months, Median, 95% CI[§]	66 (37.7-94.2)	66 (0-141.1)	45 (21.7-68.2)	82 (46.0-117.9)	0.16
TTP, months, Median, 95% CI[§]	22 (17.6-26.3)	16 (13.4-18.5)	20 (7.9-32.0)	24 (13.4-34.5)	0.47

*Log-rank test comparing OS and TTP between haptoglobin phenotypes; [§]CI=confidence interval. Hp: Haptoglobin.

Conclusion

Although our preliminary study is based on a small population with scant significant findings, we hypothesize that patients with EOC, with haptoglobin 2-2 phenotype might have longer OS and TTP because they present fewer G3 tumors and they may present a stronger immune response than do patients with 1-1 and 1-2 phenotypes. Larger studies should be conducted to assess this hypothesis.

Conflicts of Interest

The Authors declare that they have no competing interests.

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