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FROM THE DEPARTMENT OF MEDICAL RADIOBIOLOGY, KAROLINSKA INSTITUTE, AND THE DEPARTMENT OF GYNAECOLOGICAL ONCOLOGY, KAROLINSKA HOSPITAL, STOCKHOLM, SWEDEN.

DEVELOPMENT OF PLOIDY AND CELL PROLIFERATION IN SERIAL SAMPLES OF ASCITES FROM PATIENTS WITH OVARIAN CARCINOMA

K. SAHNI, B. TRIBUKAIT and N. EINHORN

Abstract

Repeated samples of ascites were obtained from 14 ovarian carcinoma patients over a period of 10 to 360 days. The samples were studied by means of flow-cytometry and monoclonal antibodies specific for bromodeoxyuridine. Ploidy and proliferation of tumor cell populations were measured to establish the significance of these properties in the development of the tumors. Ploidy was mostly a stable feature while the proportion of S-phase values increased significantly over the passage of time. This increase is discussed in relation to the expression of selective processes towards more aggressive tumor cell lines and to the development of chemoresistance.

Key words: Ovarian carcinoma, ascites, flow-cytometry, Brd-Urd labelling index, ploidy, proliferation, chemotherapy.

Tumor progression has been described as increased aggressiveness and more malignant behavior of the tumor over a passage of time (1). As a mechanism of the change to more malignant behavior, a sequential selection of variant subpopulations with survival of the fittest has been proposed (2). This has been demonstrated by performing serial chromosomal investigations in individual cases of lymphoma and leukemia where more marked changes in the chromosomes were noticed in advanced than in early stages of the disease (3). Karyotype investigations during tumor progression have been performed in solid tumors too (4). In ovarian carcinoma cellular differentiation, proliferation as well as karyotype investigations have been described in two single patients (5, 6).

Despite the widely accepted concept of clonal development of tumors, considerable heterogeneity could be observed in the tumors of various sites (7, 8). The aim of the present study was to establish possible changes of ploidy and proliferation patterns in patients with advanced ovarian carcinoma who were under therapy and to discuss these results in relation to the development of disease and to treatment.

Material and Methods

Patients. A total of 51 samples of ascites were obtained from 14 ovarian carcinoma patients at different intervals ranging from 10 to 360 days. Two samples were collected from 6 patients and more than two serial samples from 8 patients. On an average 6 months had elapsed between the development of the ascites and the collection of the first sample.

At the time of obtaining samples, treatment, status of the disease, age, menopausal status, tumor stage, and histopathology were registered (Table). The status of the disease was classified into regressive (regression in tumor size or disappearance of ascites or pleural effusion), progressive (rapid appearance of ascites or pleural effusion with shorter intervals, increase in size of the tumor or appearance of new lesions) or stable disease where none of these criteria were present.

The average age of these patients was 55 years, ranging from 33 to 77 years, with 10 patients postmenopausal. According to the Cancer Committee of the International Federation of Gynaecology and Obstetrics (FIGO), one patient was in stage 1a, one in 2b, seven in stage 3 and five in stage 4. All tumors were classified according to the WHO criteria. Epithelial tumors were the commonest. Of all epithelial tumors 10 were serous adenocarcinomas, one mucinous adenocarcinomas and three were endometroid.

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Table

Ploidy, proportion of S-phase values and clinical data of the patients studied

Patient No.	Days	Ploidy	Mean % S-value	Status of disease	Current treatment	Previous treatment	Status
1 46 Yrs., St. 3,	1	1	6	Stable	Cyclo.	1. Hyst+BSOE+Om.Res	Alive
Cl. 1c., Well diff.	37	1	4.5	Stable	Cyclo	2. Alk+Cis+Adm/m×12	
	49	1	4.0	Stable	Cyclo.		
	56	1	3.6	Stable	Cyclo.		
	70	1	2.8	Stable	Cyclo.		
	94	1	4.6	Stable	Cyclo.		
	120	1	7.0	Stable	Cyclo.		
	240	1	8.1	Prog.	Cyclo.		
	330	1	8	Prog.	Cyclo.		
	360	1	9	Prog.	Cyclo.		
2 41 Yrs., St. 4,	1	1	6.3	Stable	1. Alk+Cis	1. Alk+Cis+Adm/m×1	Dead
Cl. 3c., Poorly	54	1	–	Prog.	+Adm/mx1		
diff.	72	1	11	Prog.	No Tt.		
	82	1	–	Prog.	No Tt.		
3 57 Yrs., St. 4,	1	1	6.1	Prog.	Cyclo	1. Expl. Lap only	Dead
Cl. 3c., Poorly	21	1	5.5	Prog.		2. Alk+Cis+Adm/mx 1	
diff.						3. Alk+Adm×3	
4 33 Yrs., St. 4,	1	1	4.5	Prog.	Alk+Cis	1. Hyst+BSOE+Om.Res	Dead
Cl. 1c., Poorly	23	1	9.1	Prog.	+Adm		
diff.							
5 77 Yrs., St. 3,	1	1	2	Stable	Depo-	1. Alk+Cis+Adm/m×6	Dead
Cl. 1 c., Poorly	15	1	5.2	Prog.	provera		
diff.	75	1.3	8.9	Prog.	Nolvadex		
6 56 Yrs., St. 3,	1	1	17.7	Prog.	No Tt.	1. Hyst+BSOE+Om.Res	Dead
Cl. 1c., Poorly	7	1	–	Prog.	No Tt.	2. Alk+Cis+Adm/m×8	
diff.	12	1	18	Prog.	No Tt.		
	15	1.8	18.7	Prog.	No Tt.		
	21	1.8	21.5	Prog.	No Tt.		
	37	1.8	–	Prog.	No Tt.		
	45	1.8	33	Prog.	No Tt.		
7 67 Yrs., St. 1 a,	1	1.1	8.4	Prog.	Cyclo.	1. BSOE+Om.Res	Dead
Cl. 1c.,	21	1.1	10.8	Prog.		2. RT To Ovarian Field	
moderately						3. Alk+Cis+Adm/m×3	
diff.							
8 73 Yrs., St. 4,	1	1.1	14	Prog.	Cyclo.	1. Alk+Cis+Adm/m×9	Dead
Cl. 1c., Poorly	34	1.1	7.3	Prog.	Cyclo.	2. Cyclo./m×1	
diff.	60	1.1	11.8	Prog.			
9 68 Yrs., St. 3,	1	1.2	23	Prog.	Cyclo.	1. Hyst+BSOE+Om.Res	Dead
Cl. 1c., Poorly	45	1.6	21.5	Prog.	Cyclo.	+Bowel Res.	
diff.						2. Alk+Cis+Adm/m×7	
10 66 Yrs., St. 3c	1	1.3	17.2	Prog.	Nolvadex	1. BSOE	Dead
Cl. 1c., grade?	60	1.3	23.4	Prog.	Cyclo.	2. Alk+Cis+Adm/m×5	
	90	1.3	27	Prog.			
11 47 Yrs., St. 3,	1	1.4	15.5	Prog.	No Tt.	1. Hyst+BSOE+Om.Res.	Dead
Cl. 3c, Poorly						2. RT To Whole Abdomen	
diff.	10	1.4	15.3	Prog.		3. Alk+Cis+Adm/m×6	
12 74 Yrs., St. 3c	1	1.6	29	Prog.	Nolvadex	1. Hyst+BSOE+Om.Res.	Dead
Cl. 2c,	7	1.6	28.8	Prog.		2. Alk+Adm/m×6	
moderately	15	1.6	32.7	Prog.		3. Act.D+ Cyclo	
diff.	21	1.6	46	Prog.		+5FU/D×5/m×2	
	30	1.6	49	Prog.			
13 74 Yrs., St. 3c,	1	1.8	8.1	Prog.	Alk+Cis	1. Alk+Cis+Adm×1	Dead
Cl. 1c., Poorly	15	1.8	11.4	Prog.	+Adm		
diff.							
14 55 Yrs., St. 4,	1	0.82+1.6	18.8	Prog.	Cyclo.	1. Hyst+BSOE+Om.Res.	Dead
Cl. 3c, Poorly	21	0.82+1.6	18.3	Prog.		2. Alk+Cis+Adm/m×7	
diff.	30	0.82+1.6	18.6	Prog.		3. RT To Pelvis	
	45	0.82+1.6	19.2	Prog.			

ABBREVIATIONS

Yrs.=years, St.=stage, Cl.=class of the tumor (FIGO classification), Hyst=Hysterectomy, BSOE=bilateral salpingo-oophorectomy, Om. Res.=omentum resection. Prog.=progression, Act D=actinomycin-D, Adm=(adriamycin) doxorubicin, Alk=alkeran, Cyclo=cyclophosphamide, Cis=cisplatinum, 5 FU=5-fluorouracil, m=month, d=day, RT=radiotherapy, DNA index 1=2c (diploid) DNA content.

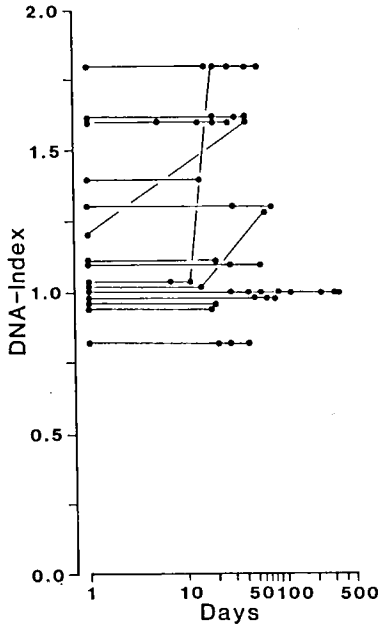

Fig. 1. Ploidy index in serially obtained samples of ascites from 14 patients with ovarian carcinoma.

Macro-radical surgery (hysterectomy + bilateral salpingo-oophorectomy + omentum resection) was carried out in eight patients. Two patients underwent bilateral salpingo-oophorectomy alone. Five patients were diagnosed only by cytology or by biopsy from metastases and fractional curettage. Two patients received external irradiation to an ovarian field and whole abdomen each respectively. Chemotherapy was given in all 14 patients (Table). At the time of reporting this all patients but one were dead.

The statistical analysis was carried out by independent t-tests.

Cell preparation. The fresh cell material was run on a percoll density gradient in order to remove erythrocytes and other inflammatory cells. The gradient was a mixture of six parts of 100% percoll (9 parts of percoll and 1 part of 1.5 mol/l NaCl) and 4 parts of 0.9% NaCl to a final volume of 10 ml and was centrifuged at 15000 rpm at 4°C for 10 min. Tumor cells were located in the part of the gradient with low density while erythrocytes and inflammatory cells were located at higher densities. The identification of the cells was done by Giemsa and Papanicolaou stains and the viability as tested by Lisamin green B stain was more than 90%.

Brd-Urd incorporation and antibody staining. The cells were after removing from gradient washed with Hanks BSS without Ca^+ or Mg^+, pH 7.2. The procedure was a modification of the methods of Dolbeare et al. (9) and Wilson et al. (10). The cells were resuspended in Hanks BSS containing 15 μmol/l of Brd-Urd, 15 μmol/l of deoxycytidine and 10% fetal calf serum and incubated for 45 min in a humidifier with 95% of air and 5% of CO_2 at 37°C, washed with Hanks BSS and fixed in ice-cold 95% ethanol. Cells were disaggregated by pipetting several times.

The overnight fixed cells were washed with Hanks BSS. After denaturating the DNA with 2 mol/l HCl for 20 min, they were washed with Hanks BSS, and incubated for 1 h in Tris buffer (Tris 0.1 mol/l, NaCl 0.07 mol/l; EDTA 0.005 mol/l, pH 7.5) containing monoclonal antibody against Brd-Urd (Partec, Switzerland) in 1:200 dilution, 0.5% bovine serum albumin and 0.5% Tween 20. After washing the cells with Hanks BSS, a second incubation was done with IgG-FITC conjugate in 2 ml of Tris for 40 min. After washing twice the cells were examined in a fluorescent microscope. The proportion of fluorescent tumor cells from more than 1000 cells in 2–3 slides from each patient was evaluated.

Flow-cytometry. Samples were centrifuged, the cell pellet was washed in Tris buffer and, after hemolysing the erythrocytes, the cells were fixed in 95% ethanol for overnight. Cells were then centrifuged, and excess ethanol removed and washed with Tris buffer containing 1 mg/ml RNAse (RNAse A, Sigma) at room temperature for 5 min. After centrifugation cell nuclei were obtained by treating the cells with 0.5% pepsin solution (Pepsin, Merck No. 7180, 1200 E/g pH 2.0) while shaking for 10 min at 37°C in water bath. They were then washed with Tris EDTA buffer, and stained with $2\times5\ 10^{-5}$ mol/l ethidium bromide in Tris EDTA buffer and measured in a flow-cytometer (ICP 11, Phywe W.G.). The output was recorded by a 256 multichannel analyser. The cell cycle composition was determined by the simplified method of Baisch et al. (11) after subtraction of background noise. The DNA content of the analysed cells was calculated in relation to the normal human lymphocytes with a coefficient of variation below 3%. Cell populations with a single G1 peak deviating less than 10% from the diploid maximum of the standard lymphocytes were considered as diploid. An aneuploid cell population was present if in addition to the diploid G1 peak another distinct maximum with corresponding G2+M peak was present or the single G1 peak deviated more than 10% from peak of the standard lymphocytes (12).

Results

The Table demonstrates the details of the 14 patients. Samples were repeated at various intervals ranging from 10 to 360 days. The first sample was obtained at a mean period of 6 months after the development of ascites.

Ploidy. Of the total 51 samples 23 (45%) were found to be diploid while 28 of them (55%) were aneuploid. Stability of the ploidy was observed in 11 patients while the ploidy changed in two of the six diploid (cases Nos. 5 and 6, Table) and in one of the 8 aneuploid (case No. 9, Table) cases. This change of ploidy in all the 3 patients was over a mean period of 45 days (ranging from 15 to 75 days, Fig. 1).

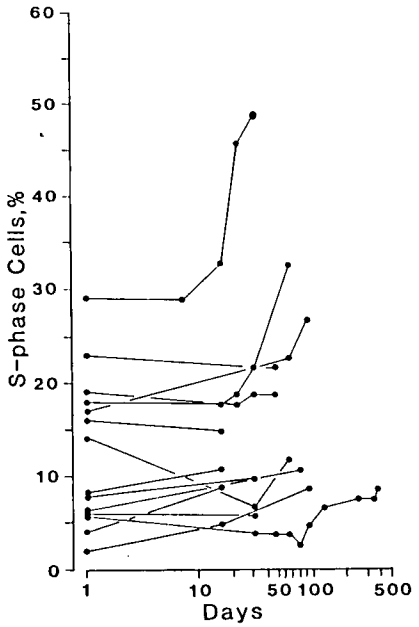

Fig. 2. Percentage S-phase values in serially obtained samples of ascites from 14 patients with ovarian carcinoma.

S-phase values. When comparing the first and the last samples obtained from the patients, a significant increase ($p=0.01$) in the proportion of S-phase values was observed over a mean period of 65 days (Fig. 2). Furthermore it was noticed that the S-phase values did not show any difference ($p=0.29$) in samples taken at shorter intervals of up to 21 days except in one case (case No. 12). In this patient there was a steep and continuous rise of S-phase values up to 30 days which was associated with rapid increase of ascites and the ultimate death of the patient.

In another patient (case No. 1) the values were relatively low and steady up to about day 240, when a rise in S-phase values was noticed. A few days later a metastatis elsewhere had appeared in this patient. Overall the mean S-phase values were 12.6 ± 7.9 and 17.3 ± 12 at the first and the last observations respectively.

Discussion

Ploidy has been demonstrated to be a stable feature in solid ovarian carcinoma (13, 14). Therefore, it has been used to predict the prognosis of ovarian carcinoma. In our series, by repeating samples of ascites over a passage of time, stability of ploidy was observed in 11 of the 14 patients. The change of ploidy in the remaining 3 patients appeared at a late stage of the disease shortly before death and was apparently not related to status of the disease, which was already progressive before the change. Thus, although a selective process of the 'survival of the fittest' might be implied in the ploidy changes, these changes lacked clinical significance in these patients with advanced tumor disease.

The most significant observation at repeated measurements of the ascites was the increase in the S-phase values in most of the patients. This increase in the fractions of S-phase cells which could also be estimated from DNA histograms was not due to the appearance of 'resting' cells in S-phase. This was demonstrated by Brd-Urd labelling index as used in the present study (15).

One explanation for the increase in the Brd-Urd labelling index could be that the time for traverse through S-phase is prolonged, resulting in an increase in the proportion of cells in the S-phase. Such an increase of S-phase duration has been observed in ascitic tumors of animals at the plateau phase of the growth (16). A decrease in the intensity of fluorescence labelled cells after Brd-Urd incorporation with time, however, could not be observed by eye in this material.

Another explanation for the increase in labelling index might be a real increase in proliferation by triggering a larger proportion of G_1 cells into S-phase. In the few cases (2 diploid and 1 aneuploid) in which the degree of ploidy changed, no increase in the S-phase values was observed. As found earlier diploid tumors have a significantly lower proportion of S-phase cells than aneuploid ones (15). Thus, the increase in proliferation rate is not necessarily linked to gross chromosomal changes detectable by measurement of ploidy. The interesting question arises if alterations of the genome expressed by small changes in the chromosomal composition not detectable by DNA studies or at the molecular level are expressed by an increase in proliferation. Since all patients in this study had been or were under chemotherapy, increase in proliferation could be a sign of the development of drug resistance. Drug resistance can be achieved by selection of a cell clone not responsive to drug treatment, after the elimination of sensitive clones or by altered mechanisms of intracellular accumulation of tumoricidal drugs. These changes may be due to changes at the molecular level, such as gene amplification (17–19). Drug resistant cells have been described to proliferate at a higher rate than the original drug sensitive tumor cells (20). The significance of high proliferation rate for the outcome of the disease in a number of tumors from other sites has been recognized previously (8, 12, 21–23).

In conclusion, we found a significant increase in the fraction of S-phase cells with duration of the tumor disease. The clinical significance of this increase and its implications for therapy remain open questions in these patients with advanced disease.

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Request for reprints: Prof. B. Tribukait, Department of Medical Radiobiology, Karolinska Institute, P.O. Box 60202, S-104 01 Stockholm, Sweden.

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