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MORPHOMETRIC APPROACH TO IMMUNOHISTOCHEMISTRY: CARCINOEMBRYONIC ANTIGEN (CEA) IN OVARIAN TUMOURS

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ABSTRACT

21 serous and 13 mucinous cystadenocarcinomas and 10 benign mucinous cystadenomas were investigated by immunohistochemical peroxidase method for carcinoembryonic antigen (CEA) to test the validity of this method in classification of these tumours. Sections were analysed in three different ways. 1.The whole section was considered a sample and total positivity was subjectively graded. 2. The section was sampled by 25 fields and each graded by two pairs of indexes: fraction and intensity of active epithelium and mucin. 3. The section was sampled by a point counting method (25 points in 25 fields) and points graded according to intensity of staining. Method 2 was reproducible, fast and easy to apply in practice and showed good discrimination ability and was almost as sensitive as method 1. Method 3 was less sensitive and needed much time. The results by method 1 were not in line with the other two methods suggesting that subjective general impression of amount of staining does not reliably reflect the quantity of stained material.

INTRODUCTION

A great deal of ovarian tumours produce carcinoembryonic antigen (CEA) as shown by immunohistochemical studies on paraffin embedded sections (Marchand et all., 1975, Rutanen et all., 1978). CEA staining has usually been estimated subjectively and it can be supposed with good reason that this method does not detect small changes and might be badly reproducible (Collan, 1982). This study was made in order to stan-

dardize the method of evaluation of staining and to get a comprehension of the amount of CEA in conventional tissue sections of various ovarian tumours.

MATERIAL AND METHODS

21 serous and 13 mucinous cystadenocarcinomas and 10 benign mucinous cystadenomas were investigated by immunoperoxidase (PAP) method, modified from that presented by Heyderman and Neville (1977). 5µm sections of formalin fixed paraffin embedded material and Dakopatts commercial serums were used. The sections were deparaffinized with xylene, and hydrated by a transfer through ethanol to water. To destroy endogenous peroxidase and to decrease nonspesific counterstaining the sections were covered with 5% H_2O_2 in distilled water and diluted (1:20) non-immune swine serum, both for 5 min. Thereafter sections were treated with rabbit anti-CEA antibody (dilution 1:100), unlabelled swine anti-rabbit antibody (dilution 1:20) and rabbit peroxidase anti-peroxidase immunocomplex (dilution 1:20) each of these for 15 min. Between each step the slides were washed with phosphate buffered saline (PBS) for 15 min. After these serum incubations the diaminobenzidine-hydrogen peroxide reaction was done by covering the slides with a solution containing 5mg DAB and 10µl 30% H_2O_2 in 10ml Tris-HCl buffer pH 7.6. The reaction time was 5min and after that the sections were washed in water.The sections were counterstained with Harris' hematoxylin, washed, dehydrated, cleared and mounted. In the staining method for controls (each section had a control) rabbit anti-CEA antibody was replaced by normal rabbit serum. The slides were investigated together with the corresponding control slide.

The stained sections were analysed with the idea of getting a quantitative estimate of the amount of CEA in the sample.First the whole section was considered a sample and total positivity was subjectively graded as negative (0), slight (1), moderate (2) or heavy (3). The heavy staining corresponded the staining intensity of CEA reached in colorectal carcinomas.

In the second place the sections were sampled at 10x objective and 10x ocular magnification by 25 randomly selected square fields determined by a 25-point ocular grid covering an area of 500µm x 500µm on the section and each field graded for area fraction of active epithelium (total area of the

epithelium as the reference) and staining intensity of active epithelium. The area fractions of the active epithelium had three categories: 25% or less (average 12.5%), 25-50% (average 37.5%) and more than 50% (average 75%). Positive staining was usually seen in the apical parts of the cells and the rest of the cytoplasm was often negative. In each field the intensity of staining if weak but distinct was scored as 1, and if moderate as 2, score 3 corresponding to the staining intensity reached in colorectal carcinomas. The score of each field was attained by multiplying the proportions of active epithelium (0.125, 0.375 or 0.750) with the corresponding intensity score and volume fraction of epithelium in that field. The latter was determined by point counting. The scores of all tested fields were summed and the sum was called the epithelial field score. For mucin the proportion of positive mucin in each field was evaluated, positive mucin scored according to intensity of staining from 1 to 3, with the staining intensity of colorectal carcinoma as the reference. The score of each field was attained by multiplying the proportions of active mucin (0.125, 0.375 or 0.750) with the corresponding intensity score and the volume fraction of the sample outside epithelium and stroma. The latter was determined by point counting. The scores of each field were summed and the sum was called the mucin field score.

Third the sections were sampled by a point counting method with 25 points in 25 randomly chosen fields. The points on positively stained area of the epithelium were graded according to intensity as above. The CEA point score of the epithelium was calculated by summing up the scores of individual points. On the other hand the points falling on active mucin were counted and scored like points falling on active epithelium. The point score of mucin was attained by summing up these scores. In practice this was done by calculating the number of points with each score with a haemocounter, multiplying the numbers with the corresponding score and summing the products. The point scores of epithelium and mucin were compared with the corresponding field scores. The field scores and the point scores were about the same magnitude. The field scores and the point scores were evaluated in the same microscopic fields and the grid used was similar in both methods.

RESULTS

In Table 1 the mean values and standard deviations of different scores are seen. The mucinous cystadenocarcinomas have the highest epithelial scores followed by the serous cystadenocarcinomas and the mucinous cystadenomas. Positively staining mucin, on the other hand, is scanty in serous cystadenocarcinomas. Between the mucinous cystadenocarcinomas and cystadenomas the difference in mucin is small. Variation within each tumour group is large.

Table 1. Results of CEA immunoperoxidase staining of 10 mucinous cystadenomas, 13 mucinous cystadenocarcinomas and 21 serous cystadenocarcinomas (subjective estimate and the amount of positive staining in sections in 25 randomly selected fields estimated with the field and the point score methods). The results are expressed as mean ± SD.

Method	Mucinous cystadenomas	Mucinous cystadeno-carcinomas	Serous cystadeno-carcinomas
Subjective estimate	0.6 ± 0.9	1.8 ± 0.6	0.7 ± 0.8
Field score			
epithelium	2.3 ± 5.2	46.0 ± 69.8	21.1 ± 60.0
mucin	24.5 ± 73.4	29.9 ± 33.2	13.7 ± 30.0
Point score			
epithelium	2.7 ± 5.7	54.2 ± 94.2	22.0 ± 67.8
mucin	17.2 ± 51.6	14.4 ± 13.4	7.8 ± 17.4

In Table 2 the subjective estimation is evaluated by the field and the point score methods. Subjective scores 1 and 2 do not seem to parallel the results of the field and point score methods: this is especially clear when epithelial staining is considered. In that case the subjective weak staining gets higher mean scores than the subjective moderate staining.

Table 2. The subjective estimation compared with the scoring methods. The results are given as mean ± SD. The range of results are shown in brackets.

	Subjective estimate		
Scoring method	0	1	2
Field score			
epithelium	0	48.0 ± 93.4 (0.5 - 256.4)	40.9 ± 64.1 (0.8 - 269.3)
mucin	0	22.2 ± 36.3 (0-98.1)	41.5 ± 58.5 (0-244.5)
Point score			
epithelium	0	54.7 ± 112.1 (0-305)	45.5 ± 82.1 (0-365)
mucin	0	11.8 ± 20.2 (0-55)	23.8 ± 38.5 (0-172)

In Table 3 the results of all tumours positive in the field score method are given. The tumours are in the order of epithelial field scores. The table shows that the subjective estimation has extremely low discrimination capacity.

Table 3. All positive tumours estimated with the field and the point score methods. The cases are in the order of the epithelial field scores. Results of subjective estimation are also shown.

Case	Subjective estimation	Field score		Point score	
		Epit.	Mucin	Epit.	Mucin
1	1	0.5	0	0	0
2	1	0.8	0	0	0
3	2	0.8	2.9	2	3
4	1	1.0	0	1	0
5	2	1.5	0	2	0
6	2	2.3	11.1	2	15
7	2	3.8	0	0	0
8	2	4.5	0	6	0
9	2	4.9	0	9	0
10	2	5.3	75.6	6	45
11	2	6.1	0	3	0
12	1	11.4	98.1	4	55
13	2	12.8	15.1	17	3
14	2	17.3	244.5	19	172
15	1	18.0	0	18	0
16	2	19.9	11.6	22	12
17	2	24.0	16.1	23	13
18	2	25.9	57.8	11	22
19	2	36.9	23.4	40	18
20	2	41.9	63.0	53	31
21	2	80.3	3.0	89	0
22	2	83.6	80.6	81	24
23	2	136.3	79.5	115	48
24	1	256.4	35.3	305	16
25	2	269.3	104.4	365	46

The positive tumours have been divided into four groups as seen in the table. The mean epithelial field scores of the four tumour groups are 1.2 ± 0.6, 6.0 ± 2.5, 19.6 ± 4.4 and 129.2 ± 89.8. The mean mucin field scores are 2.3 ± 4.1, 29.0 ± 41.5, 57.5 ± 85.5 and 55.6 ± 33.5, respectively.

With the point score method the mean values for epithelium are 1.1 ± 0.9, 4.7 ± 2.8, 18.3 ± 3.9 and 149.7 ± 120.4, and for mucin 3.0 ± 5.5, 16.7 ± 23.8, 37.0 ± 60.8 and 26.1 ± 15.8 in the four tumour groups. It can be seen that the field and point score methods give very corresponding results. Three tumours show no positive epithelium with the point score method but show slight positivity by the field score method (cases 1, 2 and 7). The results referring to mucin have a good correspondence with each other in the two methods. One tumour is without any positive point scores for mucin whereas the field score method shows slight positivity (case 21).

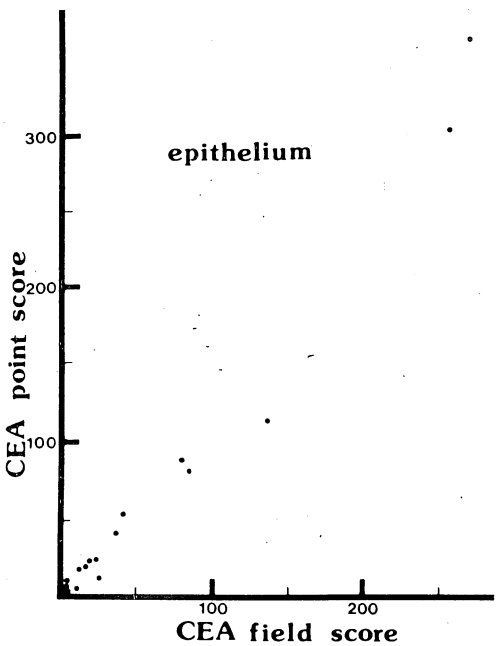

Fig 1. Correlation between the field and the point scores of the epithelium in 10 mucinous cystadenomas, 13 mucinous cystadenocarcinomas and 21 serous cystadenocarcinomas. Note that many cases are negative.

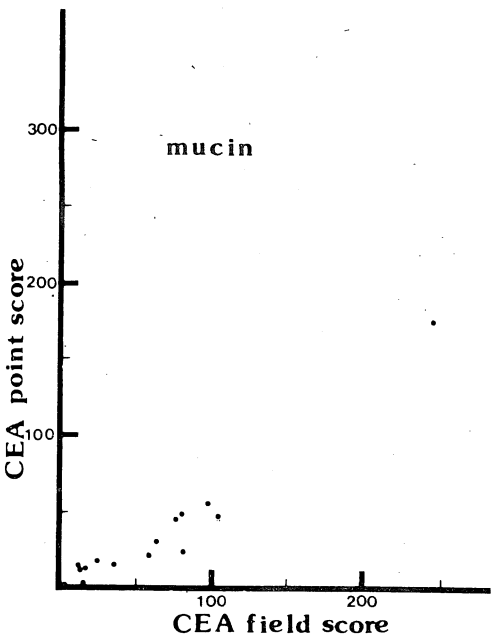

Fig 2. Correlation between the field and point scores of mucin in 10 mucinous cystadenomas, 13 mucinous cystadenocarcinomas and 21 serous cystadenocarcinomas. Note that many cases are negative.

In Figures 1 and 2 the correlation between the field and the point score methods is presented graphically. the data suggest that the point scores later increase more quickly than the field scores.

Pearson`s correlation coefficients between the field and the point scores were evaluated of all tumours positive in the field score method. The coefficients were high (epithelium 0.99, mucin 0.95) suggesting good linear correlation between the variables.

To test the reproducibility of the subjective method and the field score and the point score methods 12 samples were analysed twice in randomly selected microscopic fields. In Figures 3 and 4 it is seen that the variation between two estimations appears slightly greater with the point score method than with the field score method. The variations (in relative terms) with weak staining positivities also appear greater than with heavy staining positivities.

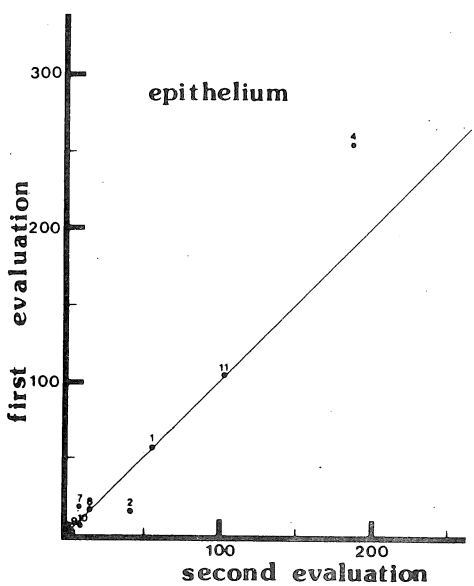

Fig. 3. The epithelial field scores of two separate estimations of 12 selected tumours. Tumours number 5,6 and 12 have score 0.

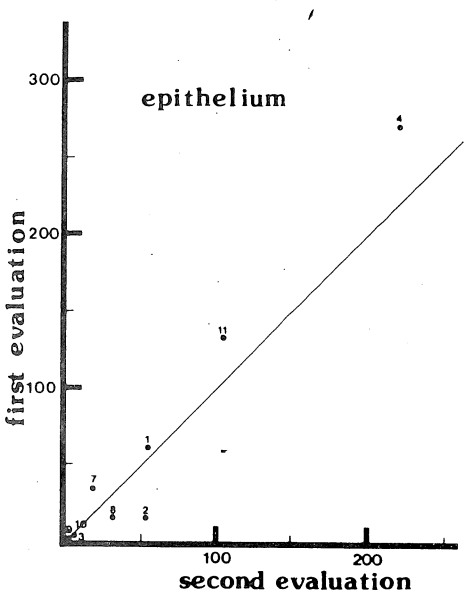

Fig. 4. The epithelial point scores of two separate estimations of 12 selected tumours.Tumours number 5,6 and 12 have score 0.

DISCUSSION

The amount of carcinoembryonic antigen can be evaluated from serum or tumour tissue of the patient. Between blood level and tissue level there may be a positive correlation (Rutanen et all., 1978) but this has not necessarily been shown in all studies. On the other hand tumour CEA concentration correlated well with immunoperoxidase staining for CEA and could be detected by immunoperoxidase technique if the tissue concentration was at least 3 µg/g (Goldenberg et all., 1976).This suggests that quantitative immunohistochemistry can be applied in the evaluation of CEA contents of the tumours.

The presence of CEA in the tumour would be of particular importance in patients who do not have elevated plasma titres, for the positive result in immunohistochemistry shows that there is a potential for secretion and raised serum values later in the course of the disease (Khoo et all., 1979). Because inflammatory conditions may cause a positive result in CEA RIA test, the immunohistochemical evaluation of CEA in the tumour itself is more reliable in that there are no false positives, if the method is correctly applied.

Subjective estimation of staining is very sensitive,because the whole sample is screened, but this method`s discrimination capacity is not good. The field score and the point score methods give results much alike each other. The results, on the other hand, differed considerably from the results of subjective estimation. There were four tumours in which the results of the field score and the point score methods were different. This shows that the field score method detects small focal CEA positivities better than the point score method. The field score method has also the advantage that it is faster. The point score method takes about 5 times more time than the field score method.

In theory the results of the field score and the point score methods should be of the same magnitude. The correlation was very good between these methods but the point scores tended to rise faster than the field scores at higher values and in fact there are methodological reasons in the latter method which make the field scores grow more slowly at high values of staining positivity (the nature of area fraction estimation).For this reason the field score method may not have a good discrimination ability with high positive values.

The better sensitivity of the field score method and the fact that it is faster than the point score method makes us recommend field scoring for the estimation of staining intensity of CEA in ovarian tumours.

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