

Immunofluorescent Assay of De Novo Vimentin Expression in Ovarian Cancer Tissues: Surgical Specimens vs Paraffin Embedded Tissue Blocks

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Abstract—The results of epithelial-mesenchymal transition analysis in serous ovarian cancer by the developed method of double immunofluorescent staining and flow cytometry while surgical tumor specimens and paraffin embedded tissue blocks are compared. When estimating de novo expression of the mesenchymal protein vimentin in epithelial tumor cells expressing cytokeratins, the results turn out to be close not only in quantitative parameters but also visually in the nature of cell distribution on the dotted plots. The results obtained indicate that the introduction of the developed new technology for the molecular diagnostics of the epithelial-mesenchymal transition of human tumors into routine laboratory practice is promising.

Keywords: immunofluorescent assay, flow cytometry, vimentin, cytokeratins, paraffin embedded tissue blocks, epithelial-mesenchymal transition

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INTRODUCTION

The epithelial-mesenchymal transition (EMT) of tumor cells is the most important molecular characteristic of malignant epithelial neoplasms, which allows us to estimate the cancer aggressiveness. In this case, the epithelial tumor cells in the EMT state acquire properties typical for cells of the mesenchymal origin [1–2], while the expression level and profile of a number of tumor proteins change [3–7], which leads to the induction of drug resistance and an increase in the metastatic potential of the cells [8–9]. De novo expression of the specific mesenchymal protein vimentin in epithelial cells is the most important marker of this molecular modification, which allows us to perform a personalized evaluation of the tumor process [10–12].

In a previous study, the data on the development of a new method for the immunofluorescent quantitative analysis of the expression of vimentin in a population of epithelial tumor cells isolated from surgical biopsy samples of solid neoplasms were presented [13]. However, during the analysis of tumor markers in the clinic, the greatest practical value consists in the possibility of conducting such an assay in the cells obtained from tumor tissue samples embedded in paraffin blocks. Considering the fact that, in this case, the preanalytical preparation is more aggressive and

includes the dehydration and rehydration of the tissue, a comparative analysis of the informational content of the developed method of the immunofluorescent assay when studying this kind of material and surgical tumor samples from the same patient was performed in this study. The subject of the study was the estimation of the epithelial-mesenchymal transition level of serous ovarian cancer by a quantitative indicator of the specific mesenchymal protein vimentin expression in epithelial tumor cells.

MATERIALS AND METHODS

The study was carried out in cells obtained from surgical biopsy samples of serous ovarian cancer and in cells isolated from samples embedded in paraffin blocks. The work included 10 comparison pairs (20 tumor samples).

The method for obtaining a single cell suspension from surgical tumor specimens. A surgical tumor specimen (up to 2 cm in diameter) was thoroughly cut with sharp scissors and incubated in a Versene solution at 37°C for 30 min. The resulting slurry was homogenized in a glass homogenizer by the gentle sixfold movement of a pestle and filtered through a 40-nm pore diameter filter (BD Falcon, United States). The cell suspension was centrifuged 5 min at 3000 rpm,

and the pellet was resuspended in a phosphate buffer solution (pH 7.4) and fixed with formaldehyde at the final concentration of 4% under vigorous shaking for 2 min (to prevent the formation of cell conglomerates).

The method for preparing cell suspensions from tumor samples embedded in a paraffin block. At least 10 tissue sections (50 nm thickness) were placed in a glass test tube (15 mL volume). To free them from paraffin, the samples were incubated thrice for 15 min in *m*-xylene. Next, the tissue was rehydrated by sequentially incubating the samples 10 min in ethyl alcohol solutions (twice in 100% and 96% alcohol and once in 80%, 70%, and 50% alcohol). Then, the samples were incubated twice for 5 min in a phosphate buffer (pH 7.4), and at the end they were centrifuged for 5 min at 3000 rpm. The pellet was cut with a sharp pair of scissors and incubated at 37°C for 30 min in a Versene solution. The resulting slurry was homogenized in a glass homogenizer by the gentle sixfold pestle movements and filtered through a 40 nm pore diameter filter (BD Falcon, United States). The cell suspension was centrifuged for 5 min at 3000 rpm, the pellet was resuspended in a phosphate buffer solution (pH 7.4) and fixed with formaldehyde at the final 4% concentration under intensive shaking for 2 min (in case the formation of cell conglomerates).

Flow cytometry. Primary monoclonal antibodies (at the final 1 : 200 dilution): mouse antibodies specific to a wide range of cytokeratins (pancytokeratins, MNF116 clone, DAKO, United States) and rabbit antibodies specific to the vimentin protein (SP20 clone, BIOCARE, United States) were used in the study. Antirabbit antibodies conjugated with the fluorescent dye DyLight650 (ab98510, Great Britain) and antimouse antibodies conjugated with DyLight488 (ab98637, Great Britain) at the final 1 : 1000 dilution and 1 : 120, respectively, were used as the secondary monoclonal antibodies. To remove debris and erythrocytes from the analysis, DNA dye Hoechst 33258 (Sigma-Aldrich, United States) was used at the concentration of 1.2 µg/mL. Only cells with stained nuclei were included in the analysis; cell conglomerates were removed from the analysis by additional gating.

Fluorescence was measured on a Navios flow cytometer (Beckman Coulter, United States). The fluorescence signal of the dyes DyLight488, DyLight650, and Hoechst 33258 was recorded in the FL-1, FL-6, and FL-9 channels, respectively. To visualize the cells distribution depending on the intensity of the fluorescence in different channels on a flow cytometer, we used the dotted plots constructed using the WinMDI 2.9 program. The level of de novo vimentin expression in the epithelial cells was determined as the ratio (%) of the number of cells coexpressing cytokeratins and vimentin to the total number of tumor cells expressing cytokeratins.

The paired Student's *t*-test included in the GraphPad Prism 7.0 package (GraphPad Software, United

States) was used for the statistical processing of the obtained results. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

When analyzing the results obtained, it is necessary to note the most important methodological details that were worked out at the stage of preparing a single cell suspension from the tumor tissue, which was embedded in a paraffin block. Firstly, we showed that the optimal section thickness is 50 µm, since the yield of intact cells suitable for the analysis on a flow cytometer sharply decreases with thinner sections, and the duration and folds of incubation with *m*-xylene for completely dissolving paraffin significantly increase with an increase in the thickness. This is a fundamentally important detail, since any complication of the preanalytical stage, as well as the insufficient number of tumor cells included in the analysis, reduce its accuracy. Another detail, intensive shaking of the suspension for 2 min while fixing the cells with formaldehyde, also contributes to the increase in the yield of intact cells. If this fixation condition is met inaccurately, a significant cells portion form dense conglomerates, which are not included in the analysis and also disrupt the operation of the flow cytometer.

The comparative assessments results of the epithelial-mesenchymal transition level in ovarian cancer tissue while studying surgical specimens and paraffin blocks of the same tumor are presented in Table 1. It can be seen that tumors of different localizations differ significantly in the level of vimentin coexpression in epithelial tumor cells expressing cytokeratins. When examining surgical specimens, the indicator fluctuated within the group on average from 22 to 70%, and in the blocks from 19 to 63% (sample nos. 1 and 7, respectively).

The differences between the parameters within one tumor when studying the paraffin block and the surgical specimen were insignificant and only in 2 out of 10 cases reached 10%. In the rest of the compared pairs, the results turned out to be practically the same, and generally within the group they were statistically insignificant when assessing the differences by the paired Student's *t*-test ($p = 0.09$).

The insignificant differences between the compared groups, revealed as a result of the analysis, confirm the importance of these methodological details: strict control of the thickness of the section paraffin block and the related conditions of incubation of the tissue sample in *m*-xylene. Otherwise, an increase in the aggressiveness of the preanalytical material preparation of the material or excessive contamination of the suspension with destroyed cells can distort the molecular aggressiveness indicator in the studied tumor.

Examples of the dotted plots obtained in the study of tumor surgical specimens and tumor material

Table 1. The level of epithelial-mesenchymal transition of cells in serous ovarian cancer studying vimentin expression in epithelial tumor cells obtained from surgical specimens and paraffin blocks

Number of tumor sample	Level of vimentin coexpression in epithelial tumor cells expressing cytokeratins, %		Differences between surgical specimen and paraffin block, %*	<i>p</i> **
	surgical specimens	paraffin blocks		
1	22	19	3	0.09
2	57	48	9	
3	38	48	↑10	
4	45	42	3	
5	43	36	7	
6	37	38	1	
7	70	63	7	
8	28	25	3	
9	58	48	↓10	
10	25	22	3	

* The difference between the indicators of vimentin expression (%) when studying the surgical specimen and paraffin block: ↓ and ↑ indicate a decrease and increase in the indicator, respectively; ** is the paired Student's *t*-test between the "surgical specimen" and "paraffin block" groups.

embedded in the paraffin blocks are shown in Fig. 1. Detailed information on the construction of the dotted plots, the boundaries of the division into quadrants, and the quantitative estimation of the epithelial-mesenchymal transition level of the tumor is given in a previous publication [13]. The informative value of the results obtained by the immunofluorescent analysis of the tumor material from the surgical specimens and paraffin blocks was evaluated by comparing the proportion of cells coexpressing cytokeratins and vimentin (double immunofluorescent staining) in the total population of the epithelial cells of the studied tumors.

Figure 1 shows that the results are close to each other not only in quantitative terms but also visually in the nature of the cell distribution in the dotted plots. Thus, the obtained data demonstrate the possibility of using not only surgical specimens but also paraffin embedded tissue blocks for the molecular diagnostics of the epithelial-mesenchymal transition by the method of double immunofluorescent analysis. Earlier, similar results were obtained when studying expression of ERCC1, a marker of DNA excision repair [14].

This observation is important for the practical use of the developed method for molecular diagnostics since it expands the prospects for the analysis when obtaining material from remote clinics. Moreover, the possibility of using tumor material from paraffin blocks allows us, if necessary, to verify the molecular diagnosis. Finally, the use of archive tumor material

allows conducting retrospective research and assessing the correlations with the clinical characteristics of the disease course or response to drug therapy.

However, when choosing a material for an immunofluorescent assay of tumor marker expression using flow cytometry, we should prefer surgical specimens since the preanalytical preparation of paraffin blocks is more laborious and aggressive. At the same time, cases of the target damage, which can change its accessibility not only for specific but also for nonspecific interaction with monoclonal antibodies, cannot be excluded. When using double immunofluorescent staining, two groups of proteins (cytokeratins and vimentin) serve as the object of the study; hence, the probability of an error during routine testing may increase.

In conclusion, it is important to again note the similarity of the quantitative indicators of the epithelial-mesenchymal transition level when studying different types of tumor material (surgical specimens and tissues from paraffin blocks). The data were obtained when evaluating the de novo coexpression of the mesenchymal protein vimentin in epithelial tumor cells expressing cytokeratins using the previously developed method of double immunofluorescent staining and flow cytometry, which indicates the prospects of introducing this new molecular technology into routine laboratory practice.

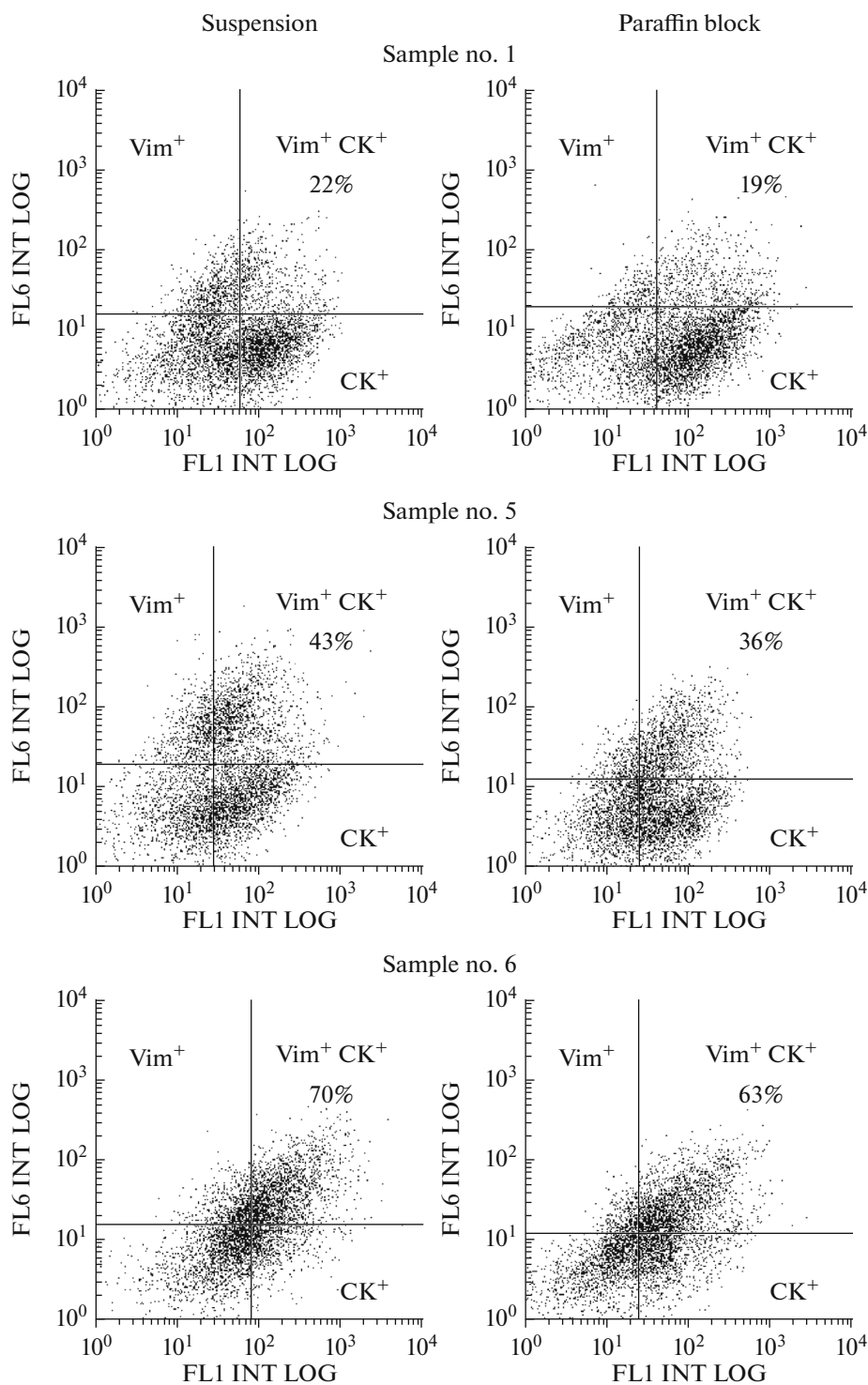


Fig. 1. Dotted plots of the cell distribution by the intensity of cell fluorescence during sequential double staining of ovarian cancer cells with antibodies to epithelial cytokeratins (CK) and to the mesenchymal protein vimentin (Vim). The abscissa shows the fluorescence intensity (Units) of the DyLight488 dye; the ordinate is the fluorescent intensity (Units) of the DyLight650 dye (designations in the quadrants: lower left, unstained cells (autofluorescence); upper left and lower right, cells expressing Vim or CK, respectively; upper right, epithelial tumor cells coexpressing Vim and CK (double immunofluorescent staining). Numbers of tumor samples correspond to Table 1.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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