Experimental Verification on the Hypothesis about the Possibility of Molecular Diagnostics of Local Tumor Spread on the Lewis Lung Carcinoma Model

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Received March 11, 2021; revised March 14, 2021; accepted March 20, 2021

Abstract—Expression of tumor-associated protein beta-III tubulin (TUBB3) was evaluated quantitatively by immunofluorescence analysis using flow cytometry in lung tissue of intact animals and on day 11 after the Lewis lung carcinoma transplantation as well in lung metastasis tissue and in visually “normal” surrounding tissue. It was shown that the tumor is characterized by a high TUBB3 expression, while also the expression of the marker was detected in both variants of visually normal lung tissue: it was lower than in tumor tissue but significantly higher than in lung tissue of intact animals. The detection of TUBB3 outside the tumor indicates that the tissue appeared to be normal has already involved in malignancy and this supports the hypothesis that tumor-associated protein TUBB3 can be used as a molecular marker of local tumor spread.

Keywords: beta-III tubulin (TUBB3), flow cytometry, Lewis lung carcinoma, immunofluorescent assay, tissue surrounding the tumor
DOI: 10.3103/S0027131421040039

The base for this study was the verification of our hypothesis: in the case of detection of the tumor-associated beta-III tubulin (TUBB3) protein in the morphologically normal tissue surrounding the tumor, this tumor marker can serve as an indicator of the local spread of the tumor process and thus an indicator of the aggressiveness of the disease.

TUBB3 belongs to the family of tubulin proteins being part of microtubules and has been intensively studied as a possible predictor of the efficacy of antitumor agents from the taxane group [1—5]. The uniqueness of TUBB3 as a potential tumor-associated marker is that it is practically not detected in normal epithelial cells [6] and is expressed only at low intensity in endotheliocytes and macrophages [7]. Concerning to normal tissue, a significant expression of TUBB3 is detected only in brain cells [8, 9] and Sertoli cells [3, 10]. As for tumors, a high expression of TUBB3 was found in the tissue of almost all nosological forms of neoplasms [11—13], which makes it very likely that this molecular marker can be efficiently used to distinguish malignant and normal cells.

In the study, the object of molecular research was the normal lung tissue of intact mice and animals with a transplanted lung tumor, since indexes of the local and distant spread of the disease can be considered as the base for the disease staging and in selecting the optimal extent of surgical resection and the treatment intensity in cancer patients. It is important that TUBB3 is not detectable in normal lung parenchyma; the proportion of endothelium and macrophages does not exceed 15% according to morphometric measurements in normal and tumor lung tissue; and the intensity of TUBB3 expression in endothelial cells is extremely low [14—16].

In this study, we studied Lewis lung carcinoma that has been widely used as an experimental model of metastasis in mice since the middle of the last century [17]. The tumor intensively metastasizes to the lungs via the hematogenous route; lung metastases are visualized at various times after subcutaneous tumor transplantation according to cells’ dose (and in 100% of dead animals) [18]. All this made it possible to implement the studies necessary to test the hypothesis about the possibility of using TUBB3 as a molecular marker...
of the local spread of the tumor process in the “normal” surrounding tissue of the organ.

EXPERIMENTAL

The study was carried out in male C57BL/6 mice weighing 22–24 g at the age of 2 to 3 months. Animals obtained from mice breeding of the experimental biological laboratory (vivarium) of the Blokhin Russian Cancer Research Center (Ministry of Health of the Russian Federation) were kept on briquetted feed with ad libitum access to water. The Lewis lung carcinoma cells (1 million in 0.5 mL of the nutrient medium 199 (PanEco, Russia)) were injected subcutaneously into the axilla of the forelimb of mice according to the standard technique [19].

The TUBB3 expression was evaluated in the following comparison groups: lung tissue of intact animals; lung tissue on day 11 after subcutaneous transplantation of Lewis lung carcinoma; and tissue of tumor metastasis in the lung and the surrounding visually normal tissue of the organ on day 24 after subcutaneous transplantation of the Lewis lung carcinoma.

A quantitative immunofluorescence assay of the TUBB3 expression parameters was performed using flow cytometry. The process of preparation a single-cell suspension from solid tissue samples, including fixation with a 4% solution of neutral formaldehyde, was described by us earlier [20].

Primary monoclonal rabbit antibodies to TUBB3 (ER15694 clone, ab52623, Abcam) and secondary anti-rabbit antibodies conjugated to the fluorescent dye DyLight 650 (ab98510, Abcam) were used for immunofluorescent staining. The final dilutions were 1 : 500 and 1 : 1000 for primary and secondary antibodies, respectively.

The cells were incubated with primary antibodies for 1.5 h in the dark at room temperature. After incubation, the cell suspension was washed with a 0.5% bovine serum albumin (BSA) solution in phosphate buffer (pH 7.4), secondary antibodies were added, and they were incubated for 1.5 h at 4°C. To remove debris and erythrocytes from the analysis after the end of incubation with secondary antibodies, the cells were incubated for 15 min with a specific DNA dye Hoechst 33258 (Sigma-Aldrich, United States) at a concentration of 1.2 μg/mL. After the end of incubation, the samples were washed twice with a BSA solution.

To control the activity of antibodies and uniformity of the experimental conditions, we used human breast cancer cell culture MCF-7 in each experiment. Two thousand cells were analyzed in the comparison groups in each experiment.

Three indicators of protein expression were examined: (1) the level of expression, namely, the percentage of specifically fluorescent cells relative to the positive control (incubation of cells with secondary antibodies only); (2) the intensity of expression, the geometric mean of the cell fluorescence minus the indicator of the positive control (arbitrary units); and (3) index, an integral indicator equal to the product of intensity and expression level, divided by 100 (arbitrary units).

The fluorescence was measured on a Navios flow cytometer (Beckman Coulter, United States) using the Navios Software. Processing of the results was carried out in FlowJo 10.0.8 (FlowJo, United States) and WinMDI 2.9 (WinMDI Software, United States) software programs.

Statistical data processing was carried out using the GraphPad Prism 6.0 software (GraphPad Software, United States). The Mann–Whitney U-test was used to assess the differences between the TUBB3 expression indicators in the comparison groups. The differences were considered statistically significant at \( p \leq 0.05 \).

RESULTS AND DISCUSSION

Results of a comparative assessment of TUBB3 expression in normal lung tissue of intact mice (1), in visually normal lung tissue on day 11 after subcutaneous transplantation of Lewis lung carcinoma (2), in the tissue of lung tumor metastasis (3), and in the “normal” tissue surrounding Lewis carcinoma lung metastasis on day 24 after subcutaneous tumor transplantation (4) are presented in Table 1. It is important to note that in order to increase the accuracy of the quantitative assessment of differences between the marker expression indicators, all comparison groups were included in each experiment as in the case shown in Fig. 1.

The first thing to note is that only insignificant expression of TUBB3 is detected in the normal lung tissue of intact animals. The average level of marker expression, i.e., the proportion of cells expressing TUBB3, was only 13.5 ± 2.9% in the normal lung tissue of intact mice, which corresponds to the published data on the proportion of endothelioocytes and macrophages expressing TUBB3 in normal lung tissue [7, 21]. It is important to note that the intensity of specific fluorescence calculated per cell turned out to be extremely low, which, as a result, affected the value of the total indicator of marker expression: the TUBB3 expression index in normal tissue was only 0.9 ± 0.6 arb. units. The latter fact also agrees with the published data on the extremely low intensity of TUBB3 expression in endothelial cells and macrophages [7, 21]. Taken together, these data indicate an insignificant contribution of TUBB3 expression in normal parenchyma to the assessment of TUBB3 as a marker of the local tumor spread in the visually normal lung tissue surrounding the tumor.

As for the tumor, the highest expression of TUBB3 (Table 1), compared to normal lung tissue, was found in the metastatic tissue of the Lewis lung carcinoma. The average expression level of the marker in the studied samples of metastasis exceeded the values for normal tis-
sue by more than 3 times (46.0 ± 8.5 vs 13.5 ± 2.9%); the expression intensity, by a factor of 18 (117.4 ± 24.7 vs 6.5 ± 2.7 arb. units); and the integral indicator of TUBB3 expression, by a factor of 60 (54.3 ± 16.4 vs 0.9 ± 0.6 arb. units). All differences are statistically significant (p = 0.03). These results are consistent with the published data on the significant expression of TUBB3 in the tissue of epithelial tumors of various nosological forms [2, 7, 11] and indicate the adequacy of the chosen experimental model to archive the set goal.

The first fact that confirmed the validity of using the tumor-associated protein TUBB3 as a marker of the local spread of the tumor process is that TUBB3

| Comparison groups | Indicators of TUB33 expression |
|-------------------|--------------------------------|
|                   | level, % | intensity, arb. units | index, arb. units |
| Normal lung tissue| 13.5 ± 2.9 | 6.5 ± 2.7          | 0.9 ± 0.6          |
| Lung metastasis of Lewis carcinoma | 46.0 ± 8.5 | 117.4 ± 24.7       | 54.3 ± 16.4       |
| Visually normal lung tissue: | | | |
| on day 11 after tumor transplantation* | 28.0 ± 3.6 | 19.5 ± 2.1       | 5.5 ± 1.3 |
| lung tissue surrounding metastasis on day 24 after tumor transplantation* | 27.0 ± 5.5 | 27.5 ± 5.1       | 7.5 ± 2.6 |

* Subcutaneous transplantation of Lewis lung carcinoma.

Fig. 1. Expression of beta-III tubulin (TUBB3) in normal, visually “normal” lung tissue, and the Lewis lung carcinoma pulmonary metastasis tissue. The abscissa is the fluorescence intensity (arbitrary units), the ordinate is the number of cells; filled histograms, after incubation with secondary antibodies only (control); transparent histograms, after incubation with antibodies to TUBB3. Numbers in the figure designate TUBB3 expression level (%) and the TUBB3 expression index (in brackets).
detected in the visually normal tissue surrounding the metastatic node in the lung (Table 1). At the same time, the marker expression indicators significantly exceed the values of the normal lung tissue of intact animals: the average level of TUBB3 expression by a factor of approximately 2 (27.0 ± 5.5 vs 13.5 ± 2.9%); intensity, more than 4 times (27.5 ± 5.1 vs 6.5 ± 2.7); and expression index, by a factor of 8 (7.5 ± 2.6 vs 0.9 ± 0.6). All differences are statistically significant (p = 0.03).

The second observation shows that cells expressing TUBB3 are also detected in the visually normal lung tissue on day 11 after subcutaneous transplantation of a highly metastatic Lewis lung carcinoma (Table 1). The expression indicators were similar to those in the visually normal lung tissue surrounding the metastasis formed on day 24 (p > 0.05) and significantly exceeded those for the normal lung tissue of intact mice. The average level, intensity, and index of the TUBB3 expression in the visually normal lung tissue were higher by a factor of approximately 2, 3, and 6, respectively, than those in normal lung tissue. In all cases, the difference was statistically significant (p = 0.03).

Figure 1 shows the real results of one of the typical experiments for the comparative evaluation of the TUBB3 expression in normal, visually normal lung tissue, and the Lewis lung carcinoma metastasis tissue in the lung. The insignificant background of TUBB3 expression associated with endothelial cells and macrophages is clearly visible in the normal lung tissue (Fig. 1a); in contrast, the expression of the marker in the metastatic tissue was found to be high (Fig. 1b). In the visually normal lung tissue surrounding the metastasis, or in the lung tissue on day 11 after tumor transplantation, the TUBB3 expression was essentially lower than in the tumor (histograms C and D vs B in Fig. 1). However, most importantly, all the marker expression indicators exceeded those in the norm (Fig. 1a). The difference between the integral index of TUBB3 expression in the comparison groups is especially pronounced: more than 20 times between the norm and the tumor and 7–8 times between the normal and visually normal lung tissues.

Thus, in the model experiments in mice, it has been shown that the expression of the tumor-associated protein TUBB3 is detected in less than 15% of cells in the normal lung tissue of intact animals; in this case, the intensity of the expression per cell is extremely low. This indicates an insignificant contribution of the expression of TUBB3 associated with endotheliocytes and macrophages in the lung parenchyma to the results of detecting this protein in visually normal lung tissue surrounding the tumor, i.e., in the evaluation of the expression of TUBB3 as a marker of the local spread of the tumor process.

The results obtained demonstrated that the studied experimental tumor (Lewis lung carcinoma), like most human epithelial solid neoplasms [22], is characterized by a high level of TUBB3 expression. Moreover, the high metastatic potential of the Lewis carcinoma with lung-tropism made it possible to assess the spread of tumor process in the lung tissue not only outside the formed metastasis but also in the organ without tumor signs during hematogenous metastasizing on day 11 after subcutaneous tumor transplantation. The TUBB3-expressing cells were detected in both cases of the visually normal lung tissue. The marker expression indexes were significantly lower compared to metastatic tissue but significantly higher compared to the normal lung tissue.

In summary, the results obtained confirmed the validity of our hypothesis: the detection of the tumor-associated protein TUBB3 in visually normal lung tissue surrounding the tumor can be considered as a molecular marker of malignant cells’ existence outside the tumor, i.e., a marker of molecular involvement of morphologically normal organ tissue in the tumor process.

The practical significance of such evaluation is unconditional, since an additional parameter clarifying the local spread and staging of the disease can serve as an additional reference point in selecting the optimal postoperative patient management, in particular to determine the necessity of drug therapy and its intensity.

FUNDING
This study was carried as part of a research program of the Blokhin Russian Cancer Research Center of the Ministry of Health of Russia (“Development and Assessment of the Clinical Significance of a New Technology for the Molecular Prediction of the Resistance and Aggressiveness of Solid Epithelial Neoplasms,” reg. no. AAAA-A20-120020690077-0).

COMPLIANCE WITH ETHICAL STANDARDS
Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Translated by G. Levit