THE DIAGNOSTIC VALUE OF THE P16INK MARKER FOR VERIFICATION OF TUMORS OF UNKNOWN PRIMARY SITE IN WOMEN WITH ISOLATED LESION OF INGUINAL LYMPH NODES

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Diagnóstico de valor de la marcador p16ink para la verificación de los tumores de localización primaria desconocida en mujeres con lesiones aisladas de nódulos linfáticos inguinales.

Key words: inguinal lymph nodes, cervical cancer, p16INK, ImageJ.

Abstract. The diagnostic value of the p16ink marker for verification of tumors of unknown primary site in women with isolated lesion of inguinal lymph nodes. Poslavska O.V., Shponka I.S., Babiy H.S. Carcinomas with an unknown primary site are a heterogeneous group of metastatic tumors, numbering from 3% to 5% of all malignant carcinoma phenotypes. An isolated lesion of the inguinal lymph nodes is remarkably associated with primary localization of tumors in the pelvis, anal canal, lower abdomen, lower limbs, and more towards in the reproductive system (vulva, vagina and cervix for women, penis for men). But in the absence of clinical signs of cancer after careful research, the histological biopsy of the lymph node with additional immunohistochemical staining with organ-specific markers becomes the diagnostic method of choice. A retrospective analysis of the histological, morphometric and immunohistochemical characteristics of the biopsy material of 59 patients with isolated lesions of the inguinal lymph nodes (35 women and 24 men) aged from 20 to 87 years (mean 59.26±15.86; median 62 years) was conducted. The distribution of variants of an isolated lesion of the inguinal lymph nodes in women showed 14 of 35 (40.00%) metastatic origin, of which p16ink (+/-) was partially 1 metastasis of melanoma (16.67%). After a thorough immunohistochemical study with a panel of organ-specific markers among isolated metastatic lesions of the inguinal lymph nodes of tumors in women, half of the revealed localized sites (7 out of 14) were in reproductive organs, namely: 4 p16ink (+) to squamous cervical cancers, 2 p16ink (+) to serous ovarian adenocarcinoma, 1 p16ink (-) to leiomyosarcoma of the uterus. Other localizations did not have a clear location, as they related to metastases of carcinoma from Merkel cells and melanomas, as a result of frequent reduction of the primary tumor lesion in the skin.

Reферат. Диагностическое значение маркера p16ink для верификации опухолей неизвестной первичной локализации у женщин с изолированым поражением паховых лимфатических узлов. Пославская О.В., Шпонька И.С., Бабий А.С. Карциномы с неизвестной первичной локализацией являются гетерогенной группой метастатических опухолей, насчитывающих от 3% до 5% всех злокачественных фенотипов карцином. Изолированное поражение паховых лимфатических узлов релевантно связывают с первой локализацией опухолей в малом тазу, анальном канале, нижней части брюшной полости, нижних конечностях и, в большей степени, в репродуктивной системе (влагалище, вульва и влагалище для женщин, пенис для мужчин). Но в случае отсутствия клинических признаков происхождения рака после тщательного исследования, диагностическим методом выбора становится гистологическое исследование биопсии лимфатического узла с дополнительным иммунохимическим окрашиванием органоспецифическими маркерами. В работе проведён ретроспективный анализ гистологических, морфометрических и иммунохимических характеристик биопсийного материала 59 пациентов с изолированным поражением паховых лимфатических узлов (35 женщин и 24 мужчин) в возрасте от 20 до 87 лет (средний 59,26±15,86; медиана 62 года). Распределение вариантов изолированного поражения паховых лимфатических узлов у женщин продемонстрировало 14 из 35 (40,00%) метастатических опухолей (другие 21 - лимфопролиферативные состояния, требующие
Carcinomas with unknown primary site are a heterogeneous group of metastatic tumors that account for 3% to 5% of all malignant phenotypes of carcinomas [7]. The isolated damage of the inguinal lymph nodes is associated with the primary localization of the tumors in the small pelvis, the anal canal, the lower abdominal region, the lower limbs and to a greater extent in the reproductive system (vulva, vagina and cervix for women, penis for men) [5]. However, in the absence of clinical signs of cancer after a thorough study, the diagnostic method of choice becomes a histological examination of the lymph node biopsy with additional immunohistochemical staining with organ-specific markers [3, 6].

P16ink is a specific marker for identifying HPV-dependent cervical neoplasia. Inhibitor of p16ink cyclokinase is a protein that blocks the cell cycle by terminating the retinoblastic oncogene (Rb) phosphorylation. In the cells infected with oncogenic HPV, overexpression of p16ink is determined. However, despite this, cells infected with HPV continue to proliferate because Rb is inactivated by the oncoprotein E7 of HPV. Since the immunohistochemical response to Ki-67 and p16ink highly correlates with HPV-infection, the detection of these markers is used to confirm the diagnosis in cases of suspicion of intraepithelial damage of high oncogenic risk [3]. But in the case of metastatic carcinoma of women, p16ink acquires a differential diagnostic value. According to the literature, the overexpression of p16ink is an absolute indicator of cervical carcinoma is found partially in adenoarcinomas of the ovaries, up to 95% of pancreatic carcinoma, as well as in squamous cell carcinoma of the oropharynx with HPV-dependent carcinogenesis [4, 6].

The aim is to investigate the complex of morphological, morphometric and immunohistochemical characteristics of cases of isolated metastases in the inguinal lymph nodes of female and male patients separately for the improvement of algorithms for diagnostics of the primary source; to find out the frequency of expression of p16ink in metastases without primary site in females.

MATERIALS AND METHODS OF RESEARCH

In this work, a retrospective analysis of histological, morphometric and immunohistochemical characteristics of the biopsy material of 59 patients with isolated lesion of the inguinal lymph nodes (35 women and 24 men) aged 20 to 87 years (mean 59.26±15.86, median 62 years) was performed. For the morphometric method, the Zeiss Primo Star-Axiocam ERC 5s microscope camera with the licensed ZEN 2 blue edition software was used, informative fields of view were recorded in .jpg format and processed in the ImageJ program with the definition of the perimeter, area and roundness of the nuclei, according to the technique described in previous publications [1, 2]. Immunohistochemical research was carried out in accordance with the protocols of the company TermoScientific (TS), (USA) on the basis of the morphological department of the treatment and diagnostic center of Pharmacies of the Medical Academy Ltd. (Dnipro) for the period from 2015 to 2018. In sections with a thickness of 4 microns, a lab visualization system Vision Quanto (TS, USA) with the detection of the protein chain using DAB Quanto Chromogen (TS, USA) was used. Characteristics of monoclonal antibodies that were used are listed in Table 1.

Statistical analysis of the parameters of areas, perimeters and coefficient of roundness of cells was carried out in the programs ImageJ and Microsoft Excel with the calculation of minimum, maximum, median, arithmetic mean and standard deviation. The analysis of the distribution of observation groups by average values of the cell nucleus size was carried out using the t-criterion. The statistically significant difference was considered at p<0.05.

RESULTS AND DISCUSSION

According to the algorithm for the diagnosis of tumors without primary site after the primary immunohistochemical panel all observations were divided into 4 groups, which showed differences in age and gender (Table 2).
Table 1

| Primary antibodies | Clone (dilution) | Localization of reaction |
|--------------------|------------------|--------------------------|
| Cytokeratin, Pan   | AE1/AE3(1:50)    | Cytoplasm                |
| Vimentin           | Ab-2/sp20 (1:200)| Cytoplasm                |
| CD45               | Ab-3 (1:200)     | Membrane                 |
| S100               | Ab-1 (1:100)     | Cytoplasm                |
| CA125              | Ab-1 (1:10)      | Membrane                 |
| Chromogranin A     | sp12 (1:400)     | Cytoplasm                |
| Synaptophysin      | sp11 (1:200)     | Cytoplasm                |
| Cytokeratin 7 (CK7)| RCK105 (1:100)   | Cytoplasm                |
| Cytokeratin 20 (CK20) | Ks 20.8 (1:100) | Cytoplasm                |
| WT-1               | Ab-1 (1:500)     | Nucleus                  |
| ER                 | sp1 (1:400)      | Nucleus                  |

According to Table 2, except lymphoproliferative lesions, immunophenotypes of other tumors were considered as metastases, the origin of which became a diagnostic task. Analysis of metastatic carcinomas of women revealed the positivity of the marker p16\textsuperscript{ink} in 6 out of 7 (85.71%) observations. Among those, 3 (42.86%) had moderate squamous cell differentiation (CKHMW +), 1 – low squamous cell (Fig. A-B), which, together with positive p16\textsuperscript{ink}, made the primary localization of these observations in the cervix probable, and 2 more cases had signs of serous moderately differentiated adenocarcinoma of the ovary, which also apart from p16\textsuperscript{ink} demonstrated positivity of CA125, WT-1 and CK7 (Fig. Г-Д).

Table 2

| Immune phenotypes of lesions of inguinal lymphatic nodes, n (%) | Gender n (%) | Age \( \bar{x} \pm SD \) (mean), years |
|---------------------------------------------------------------|--------------|---------------------------------------|
| Cyt. AE 1/3(-)/Vim.(+) / CD45(+)/S100(-), Lymphoproliferative states, 39 (66.10) | W 21 ± 39 (53.85) | \( 57,085 \pm 17,318 \) (58.75) |
|                                                               | M 18 ± 39 (46.15) | \( 55,722 \pm 12,351 \) (58.50) |
| Cyt. AE 1/3(+)/Vim.(-) / CD45(-)/S100(-), Metastases of carcinomas without primary site, 11 (18.64) | W 7 ± 11 (63.64) | \( 64,166 \pm 11,409 \) (64.00) |
|                                                               | M 4 ± 11 (36.36) | \( 59,750 \pm 11,324 \) (64.00) |
| Cyt. AE 1/3(-)/Vim.(-) / CD45(-)/S100(+), Metastases of melanoma, 7 (11.86) | W 6 ± 7 (85.71) | \( 61,500 \pm 22,006 \) (69.5) |
|                                                               | M 1 ± 7 (14.29) | 70                                    |
| Cyt. AE 1/3(-)/Vim.(-) / CD45(-)/S100(-), Metastases of mesenchymal tumors, 2 (3.39) | W 1 ± 2 (50.00) | 61                                    |
|                                                               | M 1 ± 2 (50.00) | 42                                    |

Note: \( \bar{x} \pm SD \) – mean value ± standard deviation, w - women, m - men.
The only case of p16\textsuperscript{ink} negative metastasis into inguinal lymph nodes in women was the carcinoma from Merkel cells that had specific features of the CK20 + (paranuclear), chromogranin +/-, synaptophysin +/-, CD117 +, TTF1- (Fig. E-Ж). Differences in the histological structure of p16\textsuperscript{ink} (+) and p16\textsuperscript{ink} (-) of metastatic carcinomas were reflected in the indicators of their morphometric study with the ImageJ program (Table 3).

Immunohistochemical profile of metastatic carcinomas of men with isolated lesion of the inguinal lymph nodes determined the similar distribution of tumor nosologies, but the negativity of the marker p16\textsuperscript{ink} in all observations. Namely: 1 out of 4 (25%) observations had low squamous cell differentiation (CKHMW +), without positive organ-specific markers, 1 – low-differentiated neuroendocrine cancer, 1 – metastasis of carcinoma from Merkel cells, 1 – seminal carcinoma (CD117 +, PLAP +, vitmentin) (Table 4).
Table 3

Indicators of morphometric study of metastatic carcinomas of the inguinal lymph nodes in women in the ImageJ program

| Types of primary ovarian tumors | Area (мcm²) ±SD | Perimeter (мcm) ±SD | Coefficient of «roundness» (parameter ImageJ) ±SD |
|--------------------------------|-----------------|---------------------|-----------------------------------------------|
| Squamous cell carcinoma (1)    | 55.480±9.929    | 29.735±3.421        | 0.790±0.046                                  |
| Serous AC of ovary (2)         | 36.077±8.156    | 22.450±2.470        | 0.810±0.126                                  |
| Carcinoma from Merkel cells (3)| 40.576±7.350    | 24.680±2.012        | 0.811±0.0542                                 |
| Lymphocytes of lymphatic node  | 16.738±2.693    | 15.993±1.541        | 0.746±0.098                                  |

\[ p(1)<0.05, \quad p(2)<0.05, \quad p(3)<0.05, \quad p(1)>0.05, \quad p(2)>0.05, \quad p(3)>0.05 \]

Note. ±SD – mean value ± standard deviation, statistically significant difference was considered in \( p<0.05 \).

In the group of melanoma metastases, women dominated (6:1), but it should be noted that, along with the standard immunohistochemical responses on melanoma S100 +, Vimentin +, such lesions of the inguinal lymph nodes also had partial cytoplasmic expression of p16\textsuperscript{ink}, but significantly lower, compared to neviuses which proves the pathway for carcinogenesis of loss or translocation of the CDKN2A gene. Morphometric indices of melanoma metastases are presented in Table 5.

Table 4

Indicators of morphometric study of metastatic carcinomas of the inguinal lymph nodes in men in the ImageJ program

| Types of tumors                  | Area (мcm²) ±SD | Perimeter (мcm) ±SD | Coefficient of «roundness» (parameter ImageJ) ±SD |
|---------------------------------|-----------------|---------------------|-----------------------------------------------|
| Squamous cell carcinoma (1)     | 55.480±9.929    | 29.735±3.421        | 0.790±0.046                                  |
| Neuroendocrine cancer (2)       | 18.735±2.075    | 16.500±1.128        | 0.839±0.052                                  |
| Carcinoma from Merkel cells (3) | 49.533±8.622    | 27.349±3.042        | 0.819±0.023                                  |
| Seminoma (4)                    | 47.651±4.619    | 32.612±4.714        | 0.841±0.076                                  |
| Lymphocytes of lymphatic node   | 16.738±2.693    | 15.993±1.541        | 0.746±0.098                                  |

\[ p(1)<0.05, \quad p(2)>0.05, \quad p(3)<0.05, \quad p(4)<0.05 \]

Note. ±SD – mean value ± standard deviation, statistically significant difference was considered in \( p<0.05 \).

The third subgroup of mesenchymal metastatic tumors included 2 cases: 1 metastasis of leiomyosarcoma in woman and 1 – mesenchymal tumor with undetermined malignant potential in man, both cases had p16\textsuperscript{ink} (-) negative status. Morphometric indicators are listed in Table 6.

Table 5

Indicators of morphometric study of metastatic melanomas into the inguinal lymph nodes in the ImageJ program

| Types of primary ovarian tumors | Area (мcm²) ±SD | Perimeter (мcm) ±SD | Coefficient of «roundness» (parameter ImageJ) ±SD |
|---------------------------------|-----------------|---------------------|-----------------------------------------------|
| Melanoma metastasis            | 31.106±6.694    | 22.376±4.113        | 0.600±0.065                                  |
| Lymphocytes of lymphatic node   | 16.738±2.693    | 15.993±1.541        | 0.746±0.098                                  |

\[ p<0.05, \quad p>0.05 \]

Note. ±SD – mean value ± standard deviation, statistically significant difference was considered in \( p<0.05 \).
Table 6

Indicators of morphometric study of metastases of mesenchymal tumors in the inguinal lymph nodes in the ImageJ program

| Types of primary ovarian tumors                      | Area (мcm²) ±SD | Perimeter (мcm) ±SD | Coefficient of «roundness» (parameter ImageJ) ±SD |
|------------------------------------------------------|-----------------|---------------------|-----------------------------------------------|
| Leiomyosarcoma (1)                                   | 35,254±12,831   | 25,168±5,643        | 0,467±0,130                                    |
| Mesenchymal tumor with undetermined malignant potential (2) | 74,293±19,595   | 33,476±4,060        | 0,816±0,123                                    |
| Lymphocytes of lymphatic node                        | 16,738±2,693    | 15,993±1,541        | 0,746±0,098                                    |

p(1)<0,05,
p(2)<0,05
p(1)<0,05,
p(2)<0,05
p(1) <0,05,
p(2)>0,05,

Note. ±SD – mean value ± standard deviation, statistically significant difference was considered in p<0,05.

CONCLUSIONS

1. Distribution of variants of isolated lesion of the inguinal lymph nodes in women demonstrated 14 out of 35 (40.00%) metastatic tumors (other 21 - lymphoproliferating states requiring phenotyping, but not finding the primary localization). Of 14 metastatic lesions in women, 6 observations (42.86%) demonstrated p16ink (+) phenotype (6 metastases of carcinomas from the reproductive system). For comparison: in men out of 24 cases, only 6 (25.00%) were of metastatic origin, of which 1 metastatic melanoma was found to be partially positive for p16ink (+/-) (16.67%).

2. After a thorough immunohistochemical study with a panel of organ-specific markers in isolated metastatic lesions of the inguinal lymph nodes in women, half of the detected localizations (7 out of 14) related to reproductive organs, namely: 4 p16 (+) – to squamous cell carcinoma of the cervix, 2 p16ink (+) – to serous adenocarcinomas of ovaries, 1 p16ink (-) – to leiomyosarcoma of the uterus body. Other locations did not have a clear site because they belonged to metastases of carcinoma from Merkel cells and melanoma due to the frequent reduction of the primary tumor cell in the skin.

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