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High Homogeneity of Mesothelin Expression in Primary and Metastatic Ovarian Cancer

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Abstract: To study the extent of heterogeneity of mesothelin overexpression in primary ovarian cancers and their peritoneal and lymph node metastases, a tissue microarray (TMA) was constructed from multiple sites of 220 ovarian cancers and analyzed by immunohistochemistry. One tissue core each was taken from up to 18 different tumor blocks per cancer, resulting in a total of 2460 tissue spots from 423 tumor sites (188 primary cancers, 162 peritoneal carcinosis, and 73 lymph node metastases). Positive mesothelin expression was found in 2041 of the 2342 (87%) arrayed tissue spots and in 372 of the 392 (95%) tumor sites that were interpretable for mesothelin immunohistochemistry. Intratumoral heterogeneity was found in 23% of 168 primary cancer sites interpretable for mesothelin and decreased to 12% in 154 peritoneal carcinosis and to 6% in 71 lymph node metastases ($P < 0.0001$). Heterogeneity between the primary tumor and matched peritoneal carcinosis was found in 16% of 102 cancers with interpretable mesothelin results. In these cancers, the mesothelin status switched from positive in the primary tumor to negative in the peritoneal carcinosis (3 cancers) in or vice versa (2 cancers), or a mixture of positive and negative peritoneal carcinoses was found (11 cancers). No such switch was seen between the mesothelin-interpretable primary tumors and their nodal metastases of 59 cancers, and only 1 mesothelin-positive tumor had a mixture of positive and negative lymph node metastases. In conclusion, mesothelin expression is frequent and highly homogeneous in ovarian cancer.

Key Words: mesothelin, immunohistochemistry, tissue microarray, ovarian cancer, heterogeneity

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The human mesothelin (MSLN) gene,, located at chromosome 16p13.3, encodes for a membranous precursor glycoprotein that is subsequently cleaved into the soluble 31 kD protein megakaryocyte potentiating factor and the 40 kD membrane-bound protein mesothelin.[1–3] Mesothelin was first described as a membrane protein expressed on normal and neoplastic mesothelial cells, but subsequent studies demonstrated a broader expression pattern.[1,4–9] The function of mesothelin is not fully understood. Mesothelin is expressed in only few normal tissues but has been found to be overexpressed in various tumor types at a relevant frequency.[4–10] Therefore, and because of its membranous location, mesothelin represents an attractive molecule for targeted cancer therapies. Several therapy types, including adaptive immunotherapy (CAR-T cells, TC-210 T cells), monoclonal antibodies (amatuximab/MORAb-009), recombinant immunotoxins (SS1P and LMB-100/RG7787), antibody-drug conjugates (anetumab ravtansine/BAY94-9343, DMOT4039A, BAY2287411, BMS-986148, and h7D9.v3), listeria monocytogene–

induced antitumor immune response (CRS-207 and JNJ-64041757), and immunocytokines (IL12-SS1) have provided encouraging data in animal models and/or clinical phase I and II trials.[11–24]

To identify tumor entities that might benefit most from antimesothelin therapies, it will be necessary to determine their mesothelin expression level in tumor cells. It is a conceptual weakness of biomarker testing in tumor biopsies, however, that a biomarker status is determined on primary tumor tissue removed during initial surgery, whereas the treatment is used to target tumor metastases, which were not analyzed. A change of the mesothelin expression status in metastases could either prevent response to therapy or—in case of a change from mesothelin negative to positive—lead to a situation where a treatable cancer would not be detected by standard diagnostic procedures. Studies analyzing the extent of heterogeneity of biomarker expression in cancer have shown that the level of heterogeneity depends on both the biomarker and the tumor type. For example, heterogeneity of high-level HER2 amplification and overexpression has been found to be minimal in breast cancer [25] and moderate in stomach, bladder, or colorectal cancer.[26–28] A high level of heterogeneity was found for ALK rearrangements in lung cancer,[29] Phosphatase and Tensin homolog (PTEN) deletion in prostate cancer,[30] and BRAF mutation in lung adenocarcinoma.[31]

To study the heterogeneity of mesothelin expression in ovarian cancer, an "ovarian cancer heterogeneity tissue microarray (TMA)" was constructed and analyzed by immunohistochemistry (IHC). From each of 220 ovarian cancer patients, this TMA contained up to 18 different samples (average 11.2) from different tumor blocks derived from the primary tumor, as well as corresponding peritoneal and/or nodal metastases.

MATERIALS AND METHODS

TMA

The ovarian cancer heterogeneity TMA was constructed from the cancers of 220 patients who underwent surgery at the Department of Gynecology of the University Medical Center Hamburg-Eppendorf between 2000 and 2010. The histologic subtype was serous in 165 (75%) cases, mucinous in 17 (8%), endometrioid in 16 (7%), malignant Mullerian mix tumor in 12 (5%), and clear cell in 11 (5%) cases. Selection criteria included ovarian cancers with multiple archived tumor-containing tissue blocks, which were preferably not only from the primary tumor but also included 1 or several blocks from peritoneal carcinosis and/or lymph node metastasis. In total, 2460 tumor blocks from 220 patients were included in this study. Up to 18 (average 11.2) tumor blocks, including 1 to 9 tumor blocks from the primary tumor, 1 to 9 tumor blocks from peritoneal metastases, and up to 9 blocks from different lymph node metastases, were available from each of the 220 patients. For TMA construction, 1 single 0.6 mm tissue core was taken from each block, resulting in a tissue microarray with a total of 2460 tissue cores. Among the 220 patients, 77 had tissue samples from the primary tumor and the peritoneal carcinosis, 53 from the primary tumor, the peritoneal carcinosis and the lymph node metastases, 45 only from the primary cancer, 25 only from the peritoneal carcinosis, 13 from the primary tumor and from the lymph node metastasis, and 7 patients had tissue samples from the peritoneal carcinosis and the lymph node metastasis. The detailed composition of the TMA is given in Supplemental Table S1, Supplemental Digital Content 1, http://links.lww.com/AIMM/A374. Tissues were fixed in a final concentration of 4% buffered formalin (ie, 10% dilution of 37% formalin stock solution) and then embedded in paraffin. The TMA manufacturing process was described earlier in detail.[32,33] In brief, one tissue spot (diameter: 0.6 mm) was transmitted from a cancer containing donor block ($\geq$70% cancer cells) in an empty recipient paraffin block. The paper is exempt from informed consent of the subjects because the use of archived remnants of diagnostic tissues for manufacturing of TMAs and their analysis for research purposes, as well as patient data analysis has been approved by local laws (HmbKHG, §12) and by the local ethics committee (Ethics commission Hamburg, WF-049/09). All work has been carried out in compliance with the Helsinki Declaration.

IHC

Freshly prepared TMA sections were immunostained on 1 day in 1 experiment. All immunostaining experiments were performed manually. Slides were deparaffinized with xylol, rehydrated through a graded alcohol series, and exposed to heat-induced antigen retrieval for 5 minutes in an autoclave at 121°C in pH 9 DakoTarget Retrieval Solution (Agilent; #S2367). Endogenous peroxidase activity was blocked with Dako Peroxidase Blocking Solution (Agilent; #52023) for 10 minutes. Primary antibody specific against mesothelin protein (mouse monoclonal, MSVA-235M, cat. #2198-235M, MS Validated Antibodies) was applied at 37°C for 60 minutes at a dilution of 1:150. For antibody validation, a second independent antibody (EPR19025-42) was also used for a comparative normal tissue analysis by using an identical protocol but a higher antibody concentration (1:75). Bound antibody was then visualized using the EnVision Kit (Agilent; #K5007), according to the manufacturer's directions. The sections were counterstained with hemalaun. Staining was usually membranous and often accompanied by less intense cytoplasmic positivity. All detectable membranous and cytoplasmic stainings were considered positive. The percentage of mesothelin-positive tumor cells was estimated in each tissue spot and the staining intensity was semiquantitatively recorded (0, 1+, 2+, 3+). The staining results were categorized into 4 groups as follows: negative: no staining at all, weak staining: staining intensity of 1+ in $\leq$70% or staining intensity of 2+ in $\leq$30% of tumor cells, moderate staining: staining intensity of 1+ in $>$70%, staining intensity of 2+ in $>$30% but in $\leq$70% or staining intensity of 3+ in $\leq$30% of tumor cells, strong staining: staining intensity of 2+ in $>$ 70% or staining intensity of 3

+ in >30% of tumor cells. For heterogeneity analysis, tumors were regrouped per tumor localization (ie, primary tumor, peritoneal carcinosis, and lymph node metastasis) into 33 categories including negative (absence of any detectable staining in all belonging tumor spots), homogeneously positive (at least weak staining in all analyzable tumor spots), and heterogeneously positive (at least 1 tumor spot positive and at least 1 tumor spot negative). Only tumor samples with at least 2 interpretable tissue spots were included into the following analyses.

Antibody Validation

A normal tissue array containing 8 samples from 8 different donors for each of 76 different normal tissue types (608 samples on 1 slide) was used for validation of IHC staining obtained by MSVA-235M by a second independent antibody (EPR19025-42). All cell types with positive stainings obtained by MSVA-235M (squamous epithelium of tonsil crypts, some colorectal epithelial cell groups, anal transitional, amnion cells of the placenta, some elements of corpuscles of Hassall of the thymus, scattered cells and groups of cells of endocervical mucosa and endometrium, epithelial cells of fallopian tube, some epithelial cells of the stomach, and respiratory epithelium) were confirmed by EPR19025-42 (Supplemental Figure 1, Supplemental Digital Content 2, http://links.lww.com/AIMM/A375).

Large Section Validation

A large section validation of mesothelin-negative and mesothelin-positive tissue spots was performed from 10 heterogeneously positive cancers.

Statistics

Contingency table analysis and χ^2 test were used to study associations between mesothelin expression and tumor phenotype.

RESULTS

Technical Results

Mesothelin IHC was interpretable in 2342 of the 2460 (95.2%) of the arrayed tumor samples. The remaining 118 tissue samples were not interpretable because of insufficient numbers of tumor cells in the tissue spot or lack of the entire tissue spot in the TMA section. All raw IHC data are summarized in Supplementary Table 2, Supplemental Digital Content 3, http://links.lww.com/AIMM/A376.

Heterogeneity Within Tumor Sites

Tumor sites with at least 2 interpretable tissue spots included 168 primary cancers, 154 peritoneal carcinoses, and 71 lymph node metastases (Table 1). Positive mesothelin expression was found in 2041 of the 2342 (87%) interpretable tissue spots, and in 372/392 (95%) of the tumor sites. Accordingly, our mesothelin IHC analysis identified only little intratumoral heterogeneity, which gradually decreased from the primary cancers (23% heterogeneously positive tumors) to the peritoneal carcinosis (12%) and the lymph node metastases (6%, $P<0.0001$). Remarkably, 32 (53%) of the 60 tumor sites with heterogeneous positivity had only tissue spots with negative or weak staining, suggesting low-level expression resulting in borderline IHC findings. The examples of immunostainings with homogeneously positive, heterogeneously positive, and negative findings are shown in Figure 1.

Heterogeneity Between Primary Tumors and Metastases

A total of 102 primary tumors with interpretable mesothelin data had matched peritoneal metastases and 59 primary tumors had matched lymph node metastases. The comparison between the primary and metastatic tumor sites is shown in Figure 2 for the peritoneal metastases and in Figure 3 for the lymph node metastases. Again, there was only little heterogeneity: virtually, all primary tumors with homogenous mesothelin expression had homogenously positive peritoneal carcinoses (95% of 75 primary cancers) or lymph node metastases (98% of 52 primary cancers). Also, primary tumors with heterogeneous mesothelin expression showed high rates of positive metastatic sites, including 100% homogeneously positive lymph node metastases and 81% (56% homogeneously and 25% heterogeneously) positive peritoneal carcinoses. Overall, there were 16 of 102 (16%) cancers where the mesothelin status changed between the primary tumor and the peritoneal carcinoses from positive to negative (3 cancers) or from negative to homogeneously positive (2 cancers), or where a mixture of mesothelin-positive and mesothelin-negative peritoneal carcinoses was found (11 cancers). However, the staining differences were often only small. For example, in 4 cases with mesothelin-negative primary cancers but mesothelin-positive peritoneal carcinoses (see ID #157, #177, #199, and #200 in Supplementary Table 2, Supplemental Digital Content 3, http://links.lww.com/AIMM/A376) the positive staining was only weak. No switch of the mesothelin status was seen between the primary cancers and the nodal metastases.

Large Section Validation

Large section validation of a total of 20 tumors containing tissue blocks from 10 ovarian cancers confirmed a heterogeneous mesothelin staining in these patients (Supplemental Figure 2, Supplemental Digital Content 4, http://links.lww.com/AIMM/A377).

DISCUSSION

The analysis of tumor heterogeneity is challenging, especially in advanced ovarian carcinomas presenting with large intra-abdominal tumor masses. In case of a tumor measuring 7 cm in diameter, the analysis of one 4 μm conventional whole section containing 3 × 2 cm of cancer tissues would only enable the analysis of 1/21,380 of the entire tumor mass all located in 1 specific area of the tumor. To cost-effectively analyze many different tumor regions, we constructed a heterogeneity TMA containing >11 cancer samples on average per patient. These samples were from different areas (different tumor blocks)

TABLE 1. Mesothelin Heterogeneity Status in Different Sites of Ovarian Cancer

Mesothelin IHC	Primary cancers	Peritoneal carcinoses	Lymph node metastasis	*P*
	n = 168	n = 154	n = 71	< 0.0001
Negative (%)	8.9	3.2	1.4	—
Heterogeneously positive (%)	22.6	11.7	5.6	—
Homogeneously positive (%)	68.5	85.1	93.0	—

IHC indicates immunohistochemistry.

including multiple areas from the primary tumor, as well as multiple different peritoneal and lymph node metastases. This enabled a comprehensive heterogeneity analysis of 2460 ovarian cancer patients by staining only 6 TMA sections. The data suggest that mesothelin expression is frequent and highly homogeneous in ovarian cancer. We had earlier used the concept of heterogeneity TMAs to study heterogeneity of ETS Transcription Factor (ERG) fusion,[49] PTEN alterations,[30] and deletions of chromosomes 3p,[68] 5q,[69] and 6q[70] in prostate cancer, amplifications of HER2, EGFR, CCND1, and MYC in gastric cancer,[71] HER2 and p53 in colon cancer,[28] and CCND1 amplification in breast cancer,[72] and EGFR copy number alterations in lung cancer.[52] In these studies, the validity of the approach had also been validated by whole section analyses.

The antibody used for this study had been validated according to the criteria of the International Working Group for Antibody Validation, which requires either a comparison of the findings obtained by 2 different independent antibodies or a comparison with expression data obtained by another independent method.[34] That all staining obtained by MSVA-235M on 76 different normal tissues were confirmed by a second independent antibody (EPR19025-42) demonstrates that our assay lacks significant cross-reactivities.

The results of this study show a high positivity rate of 82% in our series of 216 interpretable ovarian carcinomas. This is in the upper range of earlier studies describing a positivity rate of 30% to 100% for endometroid[8,10,35,36] and of 55% to 100% of serous ovarian cancer.[5,9,10,35–41] It is assumed that most of the variability of data in the literature are because of the use of different antibodies, different immunostaining protocols, and divergent criteria to categorize mesothelin immunostaining as positive or negative in these studies. The particularly high rate of positivity in our study may also be because of the excessive tissue sampling. It is well known that the use of multiple samples per tumor on a TMA leads to more positive cases.[42,66,67] However, 2041 out of 2342 tumor-containing TMA samples showed a positive staining in our study (87%), indicating that our extensive tissue sampling did not dramatically increase the positivity rate. Moreover, in a recent TMA study, we analyzed immunohistochemical mesothelin expression across > 12,600 individual tumors derived from 122 different human tumor types and virtually all normal tissues.[43] Using only a single 0.6 mm core per tumor, different subtypes of ovarian cancers ranked among the top 8 indications with most frequent mesothelin expression, including 71% positive mucinous carcinomas, 77% positive endometroid carcinomas, 83% positive clear cell carcinomas, and 97% positive serous ovarian cancers.

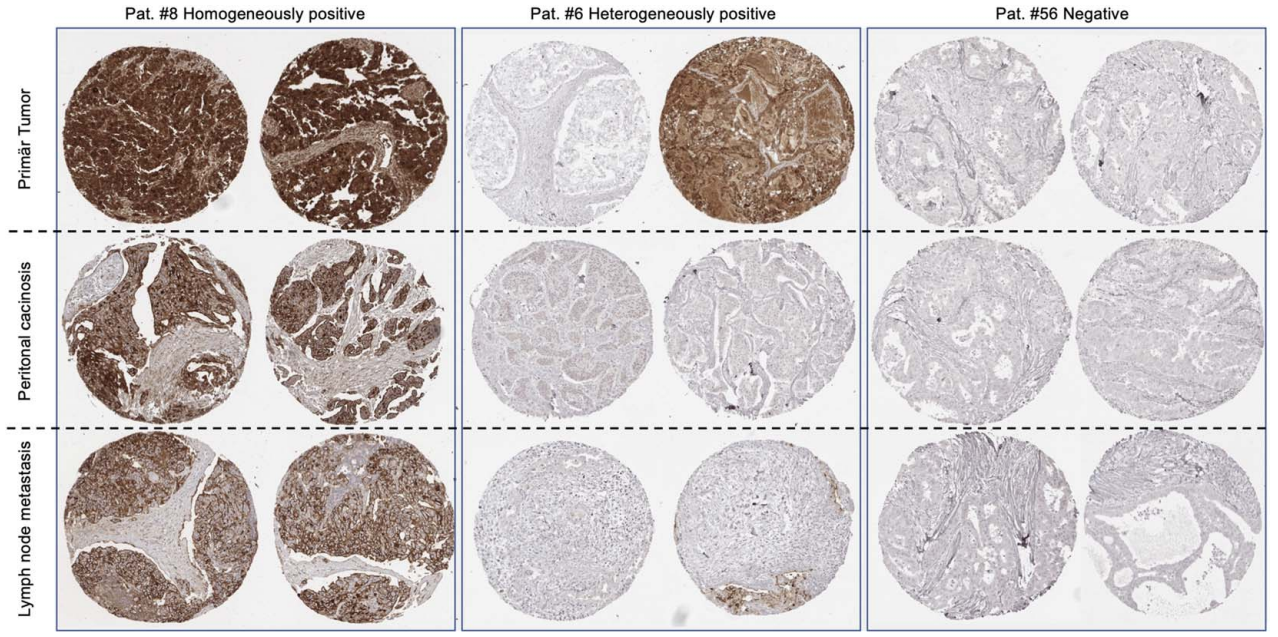

FIGURE 1. Examples of mesothelin immunostaining results in cases with homogeneous, heterogeneous, and negative findings. Pat. # corresponds to the identifier given in Supplementary Table 2.

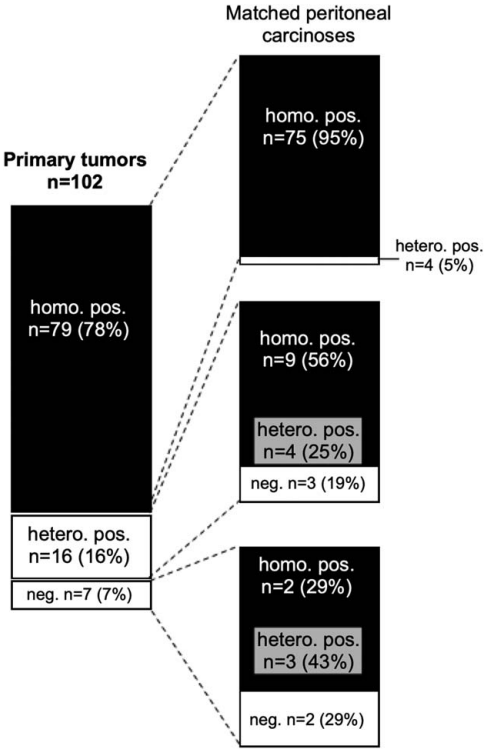

FIGURE 2. Comparison of mesothelin immunostaining results obtained from the primary tumor and the matched peritoneal carcinoses of 102 ovarian cancers.

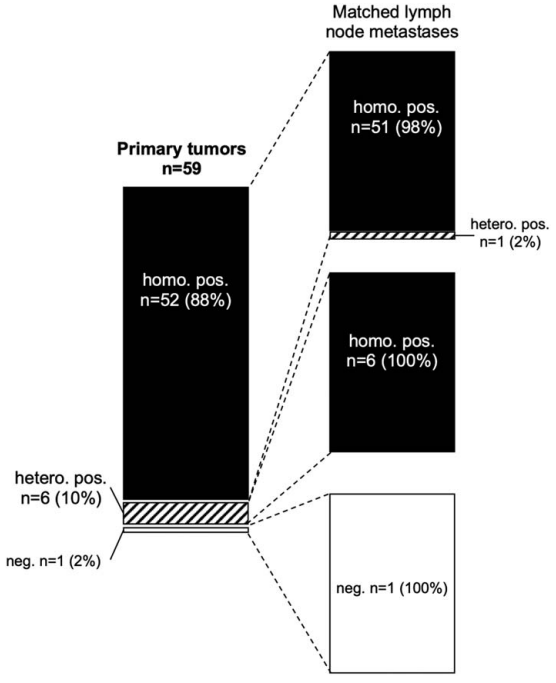

FIGURE 3. Comparison of mesothelin immunostaining results obtained from the primary tumor and the matched nodal metastases of 102 ovarian cancers.

The heterogeneity rates found within primary cancers (23%) and between the primary cancers and the peritoneal carcinosis (15%) are not neglectable but consistent with mesothelin representing a suitable therapeutic target. The intratumoral heterogeneity rate is in the range of HER2/neu amplification in breast cancer (1% to 34%)[25] and stomach cancer (5% to 75%)[44,45] or other drug targets such as EGFR mutation in lung adenocarcinoma or PIK3CA mutation in squamous cell carcinoma of the lung.[46] A similarly or even higher rate of heterogeneity was also reported for key molecular alterations of other cancers such as the 8% to 42% heterogeneous cases for *TMPRSS2:ERG* fusions in prostate cancer[47–49] or the 0% to 13% heterogeneous cases of p53 alterations in colorectal cancer.[28,50] Using a similar heterogeneity TMA approach as for this study, we had earlier reported HER2/neu heterogeneity between matched primary cancers and metastasis in 16% of breast cancers,[51] and found a much higher heterogeneity rate for prognostic alterations occurring later during cancer progression such as the 92% heterogeneous PTEN deletions in prostate cancer[30] or the 54% heterogeneous EGFR amplifications in lung cancer.[52]

Because of inherent technical issues coming along with IHC on formalin fixed tissues, we believe, that the fraction of mesothelin heterogeneous cases was rather overestimated than underestimated in this study. Some false-negative immunostaining results always occur in TMAs because not all tissues are properly fixed in all areas.[53] Unequal fixation across a tissue results in an inhomogeneous immunostaining that leads to an immunostaining gradient across a large section and will result in false-negative immunostainings, if TMA cores are taken from areas with poor reactivity.[54] Taking multiple samples per tumor, especially if this is from different tumor blocks as in this project, increases the likelihood for both detecting true heterogeneity and sampling nonimmunoreactive tissues. That heterogeneity was confirmed by large section analysis in 10 of 10 validated cases argues for a high rate of truly heterogeneous cases identified in our study, however. It seems possible that a higher degree of heterogeneity occurs in cancers with low-level mesothelin expression.

That the heterogeneity rate was higher in primary tumors than in peritoneal and nodal metastases and that many primary tumors with heterogeneous mesothelin expression developed homogeneously positive metastatic lesions would be consistent with a higher likelihood of mesothelin-positive ovarian cancer cells for peritoneal or nodal tumor spread. A higher aggressiveness of mesothelin-positive cancers would indeed be supported by functional in vitro and in vivo studies suggesting a role of mesothelin in several cancer-related cellular processes, including the PI3K/AkT and MAPK/ERK pathway.[55,56] Furthermore, increased mesothelin expression has been shown to promote cell death resistance, increased cell proliferation, invasive and metastatic properties, and angiogenesis.[55–60] In colorectal cancer, 4 studies have shown associations between high mesothelin expression and unfavorable tumor phenotype or poor prognosis.[61–64] In ovarian cancer, only 4 studies have estimated the clinical relevance of mesothelin expression.

Two of them have found a strong relationship between high mesothelin expression and shorter progression-free survival [65] and overall survival.[40] One has shown a significant association between high mesothelin expression and prolonged overall survival [39] and one found no association between mesothelin expression and overall or progression-free survival.[41]

CONCLUSIONS

Our data demonstrate frequent high level and homogeneous mesothelin expression in ovarian cancer. If antimesothelin therapies should prove efficient in the future, ovarian cancer will be an ideal cancer type for such treatments. Small biopsies are likely to be sufficiently representative for determining the mesothelin expression status of these tumors.

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