

# Diagnostic and prognostic value of Ki67 proliferation fraction in serous effusions

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**Abstract.** The Ki67 proliferation rate of mesothelial cells was determined in 20 effusions due to malignant mesotheliomas and in 20 non-neoplastic effusions, to investigate if this marker may be useful to identify neoplastic mesothelioma cells and if there is a correlation between proliferation rate and survival time.

Using the ABC-method, effusions were immunostained and the marker Ki67 was evaluated quantitatively.

Ki67 proliferation fraction showed rates from 2.3% to 70% in malignant mesothelioma cells and from 1.8% to 25.5% in reactive mesothelial cells. A significant difference was found ( $p = 0.05$ ) between those two groups. Assuming a threshold at 26%, a sensitivity of 25% and specificity of 100% resulted. Yet, due to its low sensitivity this marker seems not to be useful for differential diagnosis.

Plotting surviving period against Ki67 proliferation fraction a correlation was observed which was not significant. Long term survivors (>28 month) showed proliferation rates below 3.8%.

Unexpectedly a highly significant difference ( $p = 0.001$ ) between Ki67 proliferation rates of mesothelial cells from patients with malignant tumors other than mesothelial origin (7.0% to 25.5%) and mesothelial cells of patients without any malignant disease (1.8% to 16.3%) were observed. Setting a threshold at 10% for identification of a malignant disease, a sensitivity of 77.8% and specificity of 90.9% resulted.

## 1. Introduction

Malignant mesothelioma as a tumor of the serous membranes is still difficult to diagnose, and prognosis for patients suffering from this disease is usually poor. The first clinical symptom mostly is an effusion of the respective serous cavity (in 90%), so cytological examination is useful to diagnose this tumor minimal-invasively and early [2]. Yet, conventional cytological methods reveal malignant mesothelioma cells in serous effusions in only 45% [11]. Using adjuvant methods as DNA-image-cytometry, AgNOR-analysis and immunocytochemical markers (BerEP4, Calretinin), sensitivity and specificity increase to 61% and 99% respectively [2]. Though patients with malignant mesothelioma show surviving periods of only one to

thirteen months [3], cases of long-term survivors up to ten years are also known [13–15]. These occurring differences in survival time make it important to seek for prognostic parameters obtainable at cells in effusions.

In the first part of the following study we investigate if the Ki67 proliferation fraction can be used as a marker to distinguish between mesothelioma and non-neoplastic mesothelial cells. Secondly, we consider if a correlation between Ki67 proliferation rate of mesothelial cells may be of prognostic value.

## 2. Material and methods

### 2.1. Specimens and patient population

Subject of our study were 40 effusions of the pleural, pericardial or peritoneal cavities comprising 20 cytologically tumor cell positive (malignant epithelial mesotheliomas) and 20 tumor cell negative ones. Diagnosis of malignant epithelial mesotheliomas were

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all verified histologically. Follow ups of patients without tumor cells in effusions showed a variety of diseases: one collagenosis, two patients with a heart insufficiency, three non-Hodgkin's lymphomas, one cirrhosis of the liver, one rhabdomyosarcoma, one renal insufficiency, one embolus of the lung, one patient with multiple necrosis of the liver of unknown origin, one cholangiocarcinoma, one pancreatitis, one uterine cervical carcinoma, one chronic pleurisy, one sarcoma of the stomach, one fibroma of the ovary, one small cell lung cancer, one non-small cell lung cancer.

The material was examined in daily routine from 1997 to 2000 at the Institute of Cytopathology. The diagnosis of the smears happened as follows: Microscopic inspection of the smears allowed to differentiate between three diagnostic categories: I – tumor cell positive smears, II – tumor cell negative smears and III – smears which were suspicious for tumor cells. On smears of category III we performed (a) AgNOR-analysis, (b) immunocytochemistry and (c) DNA-cytometry. Using these methods together we finally classified the smears either as tumor cell positive or as tumor cell negative ones. Using this algorithm, a 95% correct rate of mesothelioma and 100% rate of carcinoma cell identification without false positive diagnosis resulted [11].

All specimens were taken from patients of the University Hospital of Düsseldorf as well as from surrounding hospitals. Such effusions were selected which contained at least 300 reactive or malignant mesothelial cells.

## 2.2. Staining of specimens

Native material was centrifuged (5 minutes, 300g) and subsequently decanted to separate the cells. Drops of the sediments were dispersed between slides, yielding two slides per drop coated with cells, which were immediately immersed in a fixative. As such Delaunays solution (500 ml ethanol plus 500 ml acetone (Riedel-de-Haen, Seelze, Germany) plus ten drops of 1 M trichloroacetic acid) was used. The fixation time was at least 30 minutes.

Afterwards the slides were stained according to Papanicolaou and coverslipped with Entellan.

## 2.3. Immunocytochemistry

The coverslips of the previously Delaunay fixed and Papanicolaou stained slides were first removed in xylene. The dyes were washed out with ethanol.

The immunocytochemical staining was performed by means of the Avidin–Biotin-Complex-Method (ABC).

For that purpose the slides were first heated in citric acid at 80°C for 10 minutes in a cuvette. After cooling down for 20 minutes to room temperature (RT), endogenous peroxidases were stopped by incubation in a solution of 1 ml H<sub>2</sub>O<sub>2</sub> (30% Perhydrol, Merck, Darmstadt, Germany) in 100ml methanol (Merck, Darmstadt, Germany) for 30 minutes (RT).

The H<sub>2</sub>O<sub>2</sub>–methanol-solution was removed, rinsing the slides with phosphate buffered saline (PBS, Sigma, Deishofen, Germany). They were then placed in PBS for 2 × 10 minutes. The slides were incubated with normal (horse) serum (for mouse antibodies) and horizontally placed in a humid chamber (HC) for 20 minutes (RT).

The residual normal serum was dripped off and the slides were incubated with primary antibody MIB-1 (Dianova), diluted 1 : 100 with medium (DAKO kit, DAKO Diagnostika GmbH, Hamburg) for 12 hours (RT, HC). 10 µl MIB-1 and 1 ml medium were used. Then the slides were flushed twice in TRIS/PBS-solution (50% PBS and 50% TRIS (750 ml Aqua dist., 60.57 g trichloroethylene (hydroxymethyl)amino-methan)).

According to the ABC-method the slides were now incubated with secondary (biotinylated) antibody (LINK) for 30 minutes (RT, HC) and rinsed in TRIS/PBS for 2 × 10 minutes. Then the substrate-chromogen-reagent (3-amino-9-ethylcarbazole, AEC) (Sigma, Deishofen, Germany) was applied for 40 minutes at room temperature (28 mg AEC, 7.2 ml n-n-dimethylformamide, 100 ml acetate buffer (0.1 M, pH 5.2), 106 µl H<sub>2</sub>O<sub>2</sub>).

The staining was terminated by removing the slides and flushing them with distilled water for two times five minutes. Counterstaining was performed with Mayers Haematoxylin (1 min, RT) then the slides were rinsed under tap water and coverslipped in aquatex (water based mounting medium, Merck, Darmstadt, Germany).

## 2.4. Evaluation of proliferation fraction

The Ki67 proliferation fraction of the mesothelial/mesothelioma cells in the effusions was evaluated by counting red-coloured proliferating cells in a microscope (40× objective and a 10× eyepiece). Considering only mesothelioma or mesothelial cells, 300 mesothelial cells per slide were identified morpholog-

ically as either belonging to the group “proliferation” or the group “state of rest”. Necrotic cells were not counted. Classification took place as follows: Each cell, with a recognizable red coloration of the nucleus or parts of it was rated as a proliferating cell; each cell with no such coloration was taken as a cell in state of rest.

The Ki67 proliferation fraction was given by the number of proliferating cells divided by the total number of the counted cells. To analyze the data, the *U*-test (Mann/Whitney) and the program MicroCalOrigin<sup>®</sup> were used.

### 3. Results

#### 3.1. Mesothelioma versus mesothelial cells

Effusions containing malignant mesothelioma cells showed Ki67 proliferation rates from 3.5% to 70% (average 22.4%), while tumor cell negative effusions had rates from 1.8% to 25.5% (average 10.8%). Although both groups particularly overlap within the lower range, a significant difference of the Ki67 proliferation fraction of reactive mesothelial and mesothelioma cells ( $p = 0.05$ ) occurred.

We have not observed proliferation rates higher than 26% in the group of the tumor cell negative effusions, whereas, in the group of the effusions with mesothelioma cells, proliferation rates, which were higher than 26%, occurred in every fourth sample. From these results a specificity of 100% and a sensitivity of 25% for the recognition of malignant mesotheliomas follows if a threshold of 26% is applied.

#### 3.2. Tumor cell negative effusions

In the reference group of patients without tumor cells in their effusions, we unexpectedly discovered a highly significant difference between two groups: patients with tumor cell negative effusions and no malignant disease were summarized in group A, patients with tumor cell negative effusions and known neoplastic diseases in the follow up were classified in group B. The occurring diseases were three non-Hodgkin’s lymphomas, one cholangiocarcinoma, one uterine cervical carcinoma, one sarcoma of the stomach, one small cell lung cancer, one non small cell lung cancer and one rhabdomyosarkoma.

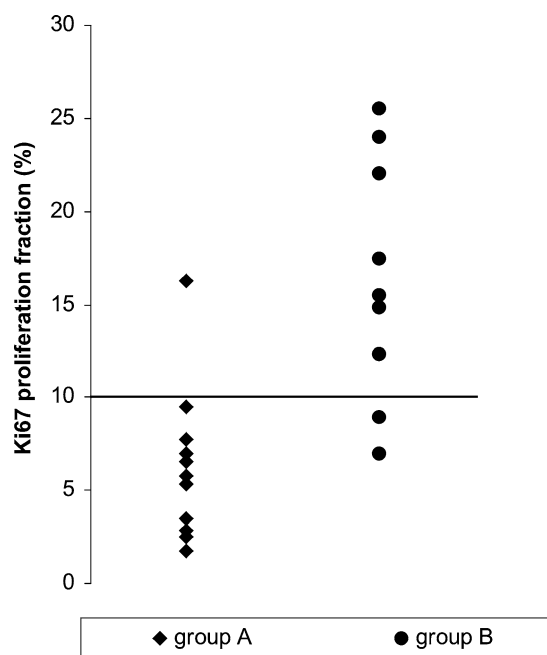


Fig. 1. Ki67 proliferation fraction (%) of reactive mesothelial cells in tumor cell negative effusions of patients without (A) and with a malignant disease (B) other than mesothelioma.

The observed Ki67 proliferation rates are illustrated in Fig. 1.

Obviously the proliferation fraction of morphologically normal mesothelial cells of patients with a neoplastic disease other than mesotheliomas is significantly higher ( $p = 0.001$ ) than the proliferation fraction of mesothelial cells from patients without a neoplastic disease. As a threshold for the identification of malignant tumors other than mesothelial origin we determined a Ki67 proliferation rate of 10%, from which resulted a sensitivity of 77.8% and a specificity of 90.9%.

#### 3.3. Proliferation rate and prognosis

Since the time of the emergence of mesotheliomas could not be determined, the surviving period of patients with this disease was defined as the period of time elapsed from cytological diagnosis to the patient’s decease (dead by any cause). From patient’s follow ups we determined an average surviving period of 10.9 month. Three patients showed clearly longer surviving periods: they lived on 28, 32 and 43 months with the diagnosis “malignant mesothelioma”. In Fig. 2 the surviving periods are plotted against the respective Ki67 proliferation fractions. We recognized a trend that the

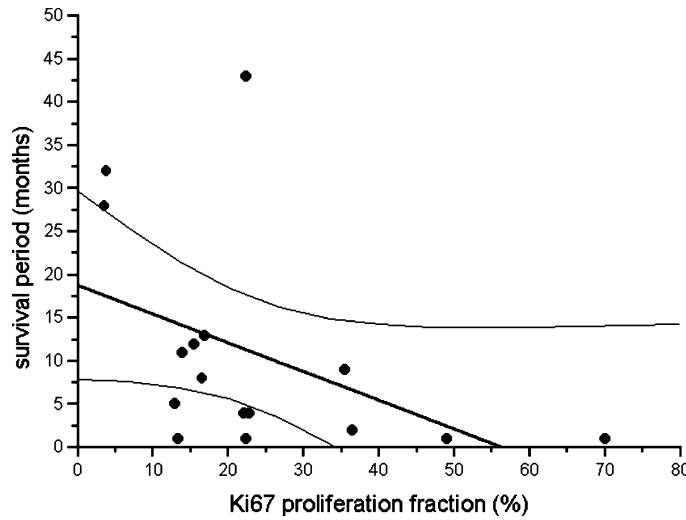


Fig. 2. Ki67 proliferation fraction (%) plotted against survival period of patients with malignant mesotheliomas, regression line and 95% confidence interval.

surviving period increases as the proliferation fraction decreases (correlation coefficient:  $-0.45$ ,  $p = 0.079$ ). We noted that two of the patients with long survival periods showed very low Ki67-proliferation-fractions of 3.5% and 3.8%.

#### 4. Discussion

Although we found a significant difference between Ki67 proliferation rates of malignant mesothelioma cells and non-neoplastic mesothelial cells ( $p = 0.05$ ) this marker seems not to be suitable for differential diagnosis of mesothelial and mesothelioma cells due to its low sensitivity. Setting a threshold of 26% proliferation rate, for example, only 25% of the malignant mesotheliomas were recognized. But when finding a very increased proliferation fraction of cells in an effusion of unknown cause this could be a hint for a malignant mesothelial tumor.

Within the reference group, we surprisingly found a highly significant difference ( $p = 0.001$ ) in the proliferation fraction of normal mesothelial cells between groups of patients with a malignant tumor at any other site in the body and patients without any malignant tumor. This allowed to distinguish between those two groups as described above: Using a threshold of 10% for identification of a malignant tumor other than mesothelial in origin a sensitivity of 77.8% and a specificity of 90.9% resulted. We cannot exclude that there is an influence on the proliferation fraction of mesothe-

lial cells by chemotherapy or radiation which some patients may have received.

Within the group of the patients without a neoplastic disease a proliferation rate higher than 10% was observed (16.5%) in only one case, which could actually indicate a malignant tumor of that patient. The particular follow up showed that he deceased suffering from a cirrhosis of the liver on the base of a chronic hepatitis C infection. It is well known, that patients who are affected with this disease develop hepatocellular carcinomas within 10 years in at least 20% of all cases [12,16]. As no autopsy was performed on this patient, we cannot exclude that this patient already suffered from an occult hepatocellular carcinoma.

Descriptions of increased proliferation rates of normal cells in the presence of malignant tumors are reported in literature. An example is given by Mott et al. (2002) describing epithelial hyperplasia found in the proximity of a malignant melanoma. There hyperplastic squamous epithelial cells showed no signs of malignancy, although irregular epithelial cords were formed [9]. Nicoulatou-Galitis et al. (2001) described similar paraneoplastic phenomena. They found hyperplastic gingiva examining patients suffering from non-Hodgkin's lymphomas, whereby the histological examination showed an inflammatory hyperplasia without malignant cells [10]. In correlation with malignant tumors of the ovaries and the uterus, Chahud et al. (2001) found hyperplasia of the uvea of both eyes [5]. Koyama et al. (1997) described hyperplasia of the squamous epithelial cells of the oesophagus as well as hyperplastic epidermal cells in the course of

acanthosis nigricans in patients suffering from carcinoma of the stomach. The tumor cells showed receptors for TGF alpha (transforming growth factor alpha) and EGF (epidermal growth factor), the serum levels of the corresponding transmitters was increased. EGF receptors were also found at the hyperplastic epidermis cells. After surgical treatment of the carcinoma of the stomach, the serum levels of TGF alpha and EGF decreased and the hyperplastic lesions of the epidermis and the oesophagus disappeared. Thus it was assumed that those transmitters, produced by the tumor, induced the observed paraneoplastic phenomena [7].

In our study we found a similar phenomenon: Normal mesothelial cells of patients with a neoplastic disease other than mesothelial in origin showed significantly increased proliferation rates. Before using the marker Ki67 diagnostically, more patients should be evaluated; but looking at the correlation we found, increased Ki67 proliferation fraction in non-neoplastic mesothelial cells could be an indicator for a malignant disease within the meaning of a cancer of unknown primary (CUP syndrome).

The prognosis for patients, who suffer from malignant mesothelioma is very bad. They have an average life expectation of 13 months in stage I and of 1 month in stage III [3]. We found similar surviving periods in our collective, which survived 10.9 months on average. But there are also examples of patients reported who lived much longer than the average. Serio et al. (2002), for example, reported surviving periods of 17 and 39 months [13]; patients are known who lived up to ten years with this disease [14,15].

The correlation between proliferation fraction of tumor cells and survival period of patients with malignant mesothelioma has already been described [4,6,8]. Thus, for instance, Beer et al. (1998) found a highly significant difference ( $p = 0.001$ ) of survival periods between patients with high or low Ki67 proliferation rates in histological slides [1].

This correlation was examined in our study as well. Analyzing the surviving period as defined above, we found a correlation between surviving time and Ki67 proliferation fraction, but it is too early to use the marker "Ki67-proliferation" for prognosis in malignant mesotheliomas just based on this trend. Yet, it is interesting to see that especially two patients with very low proliferation rates lived much longer than the average. The only patient not fitting into this scheme survived substantially longer than the average in stage I in spite of having a medium proliferation rate. This implies that additional factors will have to be taken into

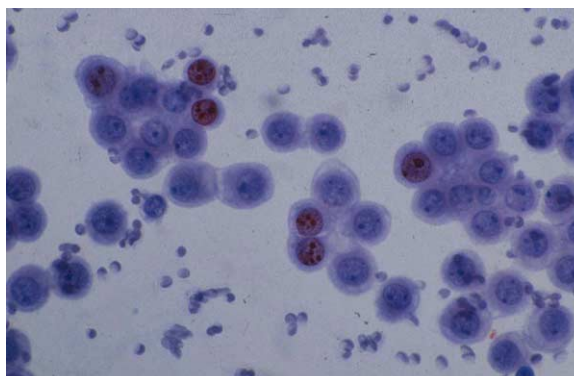


Fig. 3. ABC-method: staining of non-neoplastic mesothelial cells of a patient with an uterine cervical carcinoma with Ki67 and haematoxylin (40× objective).

account in this case to explain his long surviving period.

We realize that the patients investigated for this study show different clinical situations as they were in different stages of their disease and/or had received different treatments.

Further studies are required where patients in the same tumor stage and with a uniform therapy are investigated. Since patients with malignant mesothelioma in the final stage of their disease will decease very soon, we suggest to select mainly patients in an early stage when the prognostic relevance of proliferation rate of mesothelioma cells shall be investigated.

## 5. Conclusion

The Ki67 proliferation fraction in effusions is not suitable to distinguish between reactive mesothelial and mesothelioma cells due to its low sensitivity.

The proliferation fraction of normal mesothelial cells in tumor cell free serous effusions of patients with a malignant diseases is significantly increased. However, before using this marker diagnostically we recommend that more patients should be evaluated to confirm this observation.

We suppose a correlation between Ki67 proliferation fraction and survival time of patients with malignant mesothelioma. Further studies are required before using this marker prognostically.

## References

- [1] T.W. Beer, R. Buchanan, A.W. Matthews, R. Stradling, N. Pullinger and R.J. Pethybridge, Prognosis in malignant mesothe-

- liomas related to MIB-1 proliferation index and histological subtype, *Human Pathology* **29**(3) (1998), 246–251.
- [2] A. Böcking, H. Motherby and N. Pomjanski, Treffsicherheit der Ergußzytologie samt adjuvanten Untersuchungsmethoden, *Deutsches Ärzteblatt* **97** (2000), 2626–2630.
- [3] C. Boutin, F.G. Rey and J. Gouvernet, Malignant mesothelioma: Prognostic factors in a series of 125 patients studied from 1973 to 1987, *Bulletin de L'Academie Nationale de Medecine* **176**(1) (1992), 105–114.
- [4] S. Brozzetti, N. D'Andrea, M.R. Limiti, M.C. Pisanelli, R. De Angelis and A. Cavallaro, Clinical behavior of solitary fibrous tumors of the pleura. An immunohistochemical study, *Anticancer Res.* **20**(6C) (2000), 4701–4706.
- [5] F. Chahud, R.H. Young, J.F. Remulla, J.J. Khadem and T.P. Dryja, Bilateral diffuse uveal melanocytic proliferation associated with extraocular cancers: review of a process particularly associated with gynecologic cancers, *Am. J. Surg. Pathol.* **25**(2) (2001), 212–218.
- [6] C.E. Comin, C. Anichini, V. Boddi, L. Novelli and S. Dini, MIB-1 proliferation index correlates with survival in pleural malignant mesotheliomas, *Histopathology* **36**(1) (2000), 26–31.
- [7] S. Koyama, K. Ikeda, M. Sato, K. Shibahara, K. Yuhara, H. Fukutomi, K. Fukunaga, N. Kanazawa, K. Yuzawa, K. Fukao, T. Iijima, M. Kikuchi, T. Tomiya and K. Fujiwara, Transforming growth factor-alpha (TGF alpha)-producing gastric carcinoma with acanthosis nigricans: an endocrine effect of TGF alpha in the pathogenesis of cutaneous paraneoplastic syndrome and epithelial hyperplasia of the esophagus, *J. Gastroenterol.* **32**(1) (1997), 71–77.
- [8] E. Leonardo, F. Zanconati, D. Bonifacio and L.D. Bonito, Immunohistochemical MIB-1 and p27kip1 as prognostic factors in pleural mesothelioma, *Pathology, Research and Practice* **197**(4) (2001), 253–256.
- [9] R.T. Mott, A. Rosenberg, S. Livingston and M.B. Morgan, Melanoma associated with pseudoepitheliomatous hyperplasia: a case series and investigation into the role of epidermal growth factor receptor, *Journal of Cutaneous Pathology* **29**(8) (2002), 490–497.
- [10] O. Nicolatou-Galitis, T. Papadaki, M. Moshovi, J.J. Kamma, C. van Vliet-Constantinidou, C. Tsoumakas, A. Kattamis and F. Tzortzou-Stathopoulou, Gingival overgrowth as the initial paraneoplastic manifestation of Hodgkin's lymphoma in a child. A case report, *J. Periodontol.* **72**(1) (2001), 107–112.
- [11] N. Pomjanski, H. Motherby, B. Buckstegge, K. Knops, B.L. Rohn and A. Böcking, Early diagnosis of mesothelioma in serous effusions using AgNOR analysis, *Analyt. Quantit. Cytol. Histol.* **23**(2) (2001), 151–59.
- [12] L.B. Seeff, Natural history of hepatitis C, *Am. J. Med.* **107**(6B) (1999), 108–158.
- [13] G. Serio, A. Scattone, A. Pennella, C. Giardina, M. Musti, T. Valente and L. Pollice, Malignant decidual mesothelioma of the pleura: report of two cases with long survival, *Histopathology* **40**(4) (2002), 348–352.
- [14] G.W. Sybrecht, ed., *TIM, Chapter: Pneumologie*, Thieme, Germany, 2001, pp. 1572–1574.
- [15] C.F. Wong, S.L. Fung, W.W. Yew and K.H. Fu, A case of malignant pleural mesothelioma with unexpectedly long survival without active treatment, *Respiration* **69**(2) (2002), 166–168.
- [16] J.P. Zarski and M. Cohard, Hepatitis C, *Revue du Praticien* **45**(2) (1995), 180–184.



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