

## SURGICAL EXCISION PROMOTES TUMOR GROWTH AND METASTASIS BY PROMOTING EXPRESSION OF MMP-9 AND VEGF IN A BREAST CANCER MODEL

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Surgery is still the main curative therapeutic modality for breast cancer. Although surgery often results in the successful removal of the primary tumor, its process could increase the risk of metastases of residual cancer cells. Understanding of the connection between breast cancer metastasis and surgical wound will lead to the establishment of a proper treatment strategy for postoperative cancer patient. **Aim:** To study the influence of surgical procedure on the metastasis of primary breast cancer. **Methods:** We established MDA-MB-435 human breast cancer xenograft model. Levels of Pro-matrix metalloproteinase 9 (Pro-MMP-9) and vascular endothelial growth factor (VEGF) in host serum and tumors were tested at different time points with ELISA and zymography and correlated to tumor growth and postoperative metastasis. **Results:** Our study demonstrated surgical wound had promoting effect on tumor growth and pulmonary metastasis of human breast cells, if tumor cells remain in bodies. This effect might be related to the postoperative interaction of cancer and host cells, which resulted in expression of Pro-MMP-9. Surgical process could also increase the VEGF expression in tumor tissues. **Conclusions:** Surgical wound-produced host Pro-MMP-9 and tumor cell VEGF might be important mediators leading to metastasis of residual breast cancer after surgery. **Key Words:** surgical wound, metastasis, matrix metalloproteinases-9, breast cancer, vascular endothelial growth factor.

Breast cancer remains the most common cancer among women worldwide, with more than 1 million new case and 370,000 deaths each year [1]. Most breast cancer deaths are due to metastatic disease, not primary tumor burden [2, 3]. Surgery is still the main curative therapeutic modality for breast cancer. Although surgery often results in the successful removal of the primary tumor, the process of it could increase the risk of metastases of residual cancer cells to other organs [4]. The effect of surgery on metastasis may be attributed to a number of factors, including immunosuppression after surgical stress, action of cytokines or changes of tumor microenvironment [5]. However, the underlying mechanisms are still not fully elucidated. Understanding of the connection between breast cancer metastasis and surgical wound will lead to the establishment of a proper treatment strategy for postoperative cancer patient.

Matrix metalloproteinase 9 (MMP-9) is one of the family members that are capable to degrade almost of extracellular matrix proteins, and is closely related to tumor invasion and metastasis [6]. Recent study has shown that MMP-9 not only degrades matrix proteins, but also «nonmatrix», such as growth factors, cytokines, chemokines or death receptors [7–9], suggesting that MMP-9 may regulate both tumor microenvironment [10–12] and metastasis [13, 14]. Tumor and stromal cells can mutually induce MMPs, which in turn contribute to overall tumor invasion [15]. Clinical data has revealed that serum MMPs are related to prognosis of cancer patients [13]. Thus, objective evaluation of the correlation between MMP-9

expression level and surgical stress may provide useful marker to monitor metastasis after surgery.

Angiogenesis plays a key role in both wound healing and ability of cancer cells to survive and growth. Investigation into angiogenesis response to surgical stress may help guide surgical approaches and timing of antiangiogenic therapy. Vascular endothelial growth factor (VEGF) is one of important mediators of angiogenesis and is known to be a critical factor to stimulate tumor growth and metastasis [16–19]. Establishment of the correlation of VEGF expression level in serum or tumor cells with postoperation metastasis may provide valuable information for predicting metastasis.

To further evaluate the relations of surgery, tumor metastasis and their mechanisms, we established MDA-MB-435 human breast cancer xenograft model. Expression levels of MMP-9 and VEGF in host serum and tumors were tested at different time points with ELISA and zymography and correlated to tumor growth and postoperative metastasis.

### MATERIALS AND METHODS

**Cell lines.** Originally the human breast carcinoma cell line MDA-MB-435 was obtained from the American Type Culture Collection and maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub> in humidified air. Cells were cultured in L-15 (Invitrogen) supplemented with penicillin/streptomycin (100 IU/ml and 100 µg/ml) and 10% fetal bovine serum (FBS).

**Mice.** BALB/ca female nude mice at the age of 5 weeks (Shanghai Institute of Materia Medica, Chinese Academy of Science, Shanghai, China) were used in this study. Mice were kept in laminar-flow cabinets under specific pathogen-free conditions, cared, and handled according to the recommendations of the NIH Guidelines for Care and Use of Laboratory Ani-

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Abbreviations used: MMP-9 – matrix metalloproteinase 9; VEGF – vascular endothelial growth factor.

mals. Experimental protocol was provided by Shanghai Medical Experimental Animal Care Committee.

**Tumor model and surgical procedures.** When mice were at 6 weeks of age, tumor cells ( $6 \times 10^6$ /mouse) were injected into the right thoracic mammary fat pad of all mice to produce orthotopic primary tumors. Mice were randomly assigned to the control group ( $n = 14$ ), surgery group ( $n = 17$ ) and sham surgery group ( $n = 11$ ). Tumor resection (surgery group) involved making an elliptic incision around the tumor and dissecting it away from the chest wall. The tumor mass was gently removed and the incision closed with wound clips. Sham treatment (sham surgery group) involved making a 2-cm incision lateral to the primary tumor, leaving the primary tumor intact and closed the incision with wound clips. All surgical procedures were done under general anesthesia pentobarbital (0.34 mg of body mass) administered by i.p. injection.

**Blood serum isolation.** Blood was collected periodically by cutting off vena caudalis after surgery. After 8 min centrifugation at 2800 g, blood serum was extracted and frozen at  $-80^\circ\text{C}$ .

**Tumor protein extraction.** Tumor tissue was homogenized in lysis buffer containing 150 mM NaCl, 100 mM Tris, 1% Tween-20, 50 mM diethyl dithiocarbamate, 1 mM phenylmethylsulfonyl fluoride, 0.001  $\mu\text{M}$  aprotinin, and 1  $\mu\text{M}$  pepstatin. The mixture was stirred for 1 h at  $4^\circ\text{C}$  and then centrifuged at 2800 g for 20 min at  $4^\circ\text{C}$ . The supernatant was separated and determined by protein assay. Equal amounts of protein were applied to 12% SDS-polyacrylamide gel and separated by electrophoresis.

**Gelatin zymography.** Zymography was carried out by electrophoresis in the presence of SDS in 10% discontinuous polyacrylamide gels containing 0.1% gelatin. Samples were loaded on the gel after dilution into sample buffer ( $2 \times$ ) consisting of 60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS and 0.1% bromophenol blue. Electrophoresis was performed under non-reducing conditions at 20 mA for 3 h at room temperature. The gel was washed twice for 30 min each in 2.5% Triton X-100 to remove SDS, incubated in substrate buffer (50 mM Tris-HCl, 5 mM  $\text{CaCl}_2$ , 0.2 mM NaCl, 0.02% Brij-35, pH 7.5) for 24 h at  $37^\circ\text{C}$ . Gel was stained with 2% Coomassie brilliant blue G-250 (Pierce Rockford, IL) for 30 min at room temperature, and destained (10% ethanol, 10% acetic acid, 80% water). Zymograms were analyzed by densitometry and the quotient between the intensities obtained for pro-MMP-9 calculated, and data were presented as fold change compared to control [20].

**ELISA.** Mouse Pro-MMP-9, Mouse VEGF levels in serum and tumor extracts (50–200 g) were measured by enzyme immunoassay (R&D Systems) [21]. The serum samples were diluted to appropriate concentrations, and analyzed in duplicate. Absorbances were determined at 450 nm. Readings at 570 nm were subtracted from the readings at 450 nm to correct for optical imperfections. At the same time, a standard curve was obtained by linear regression analysis of protein concentration versus absorbance in a double logarithmic plot, basing on the standard curve to generate for each set of samples assayed.

**Histologic and immunohistochemical analysis of metastases.** At the end points, mice were necropsied and organs (including lung, brain, liver, kidney, heart, and spleen) were examined for surface metastases by microscopy ( $\times 8$ ). The whole lungs and livers from each mouse were fixed in 10% neutral-buffered formalin, embedded in paraffin, and were sectioned at every 100  $\mu\text{m}$  for 10 pieces [22]. Tissue sections were stained with H&E and examined by light microscopy for metastases.

The percent metastatic burden in the lungs was quantified by point counting method. 10 pieces of H&E-stained slide per mouse were examined using light microscopy ( $\times 100$ ) for metastases. The biggest number of metastases in the 10 pieces of H&E-stained slide was calculated as the metastatic burden of each mouse.

**Statistical analysis.** The data were expressed as the mean  $\pm$  SD. Differences between means were determined using the Student's test when groups passed both a normality test and an equal variance test.  $\chi^2$  test was used for the incidence rate of lung metastasis. Software stata 7.0 was used for correlations analysis. Significance was accepted at  $P < 0.05$ .

## RESULTS

**Surgical wound promoted pulmonary metastasis of human breast cells.** To determine whether surgical wound would stimulate metastasis of tumor cells, all mice were necropsied at the endpoint of experiments and organs (including lung, brains, liver, kidney, heart, and spleen) were examined for metastases with microscope. Superficial metastases were not observed in organs examined except for lung. Incidence of surface lung metastases occurrence was 9 of 14 (64%) in the control group and 11 of 11 (100%) in the sham group ( $P < 0.05$ , table 1). No surface lung metastases were observed in surgery group.

**Table 1.** Effects of surgical wound on the incidence of spontaneous metastases from MDA-MB-435 primary mammary tumor in nude mice

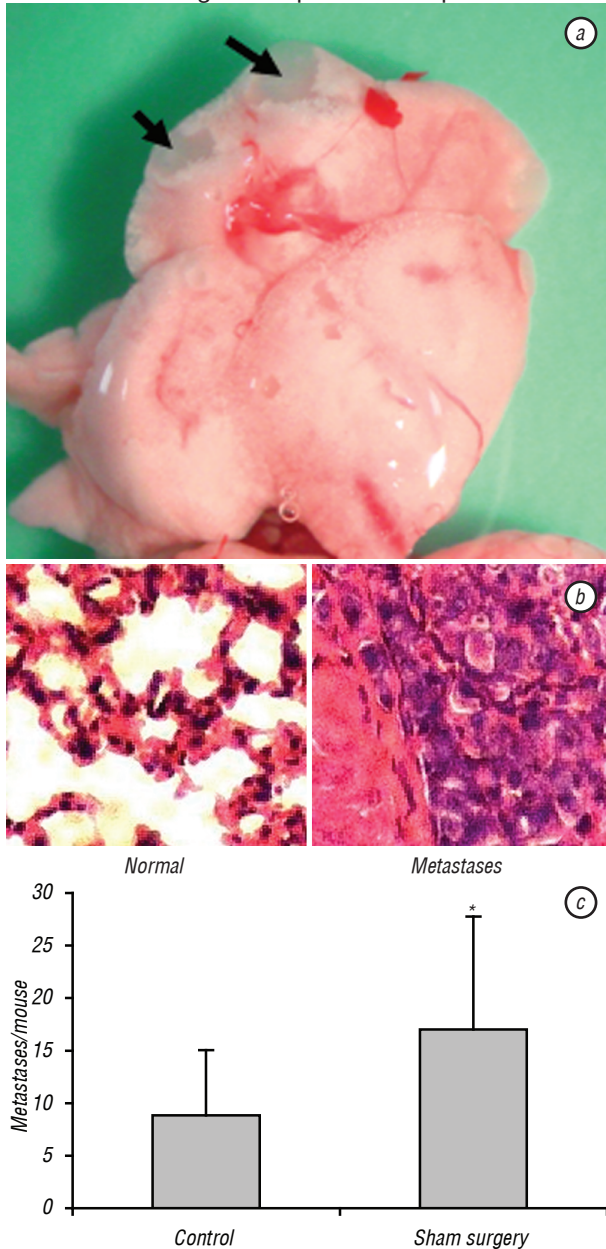
Group	Pulmonary metastasis	
	Superficial evidence	Histologic evidence
Control	9/14	14/14
Surgery	1/17	1/17
Sham surgery	11/11*	11/11

\*Statistically significant ( $P < 0.05$ ), compared with control.

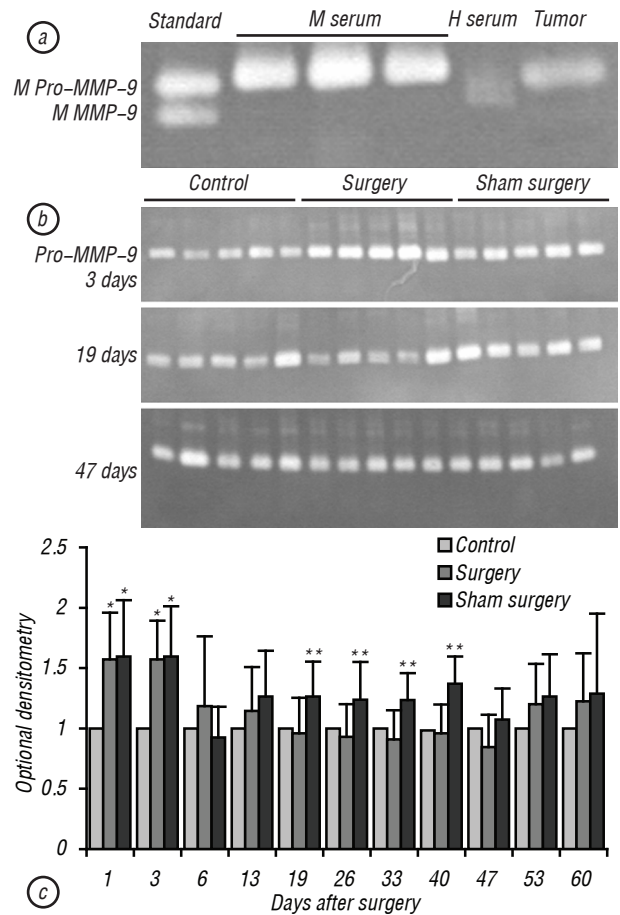
Histologic sections of lung organ were further examined by H&E staining to compare the metastatic burdens between control and sham groups. We found the number of metastasis foci per mouse ( $17.0 \pm 10.8$ ) was significantly higher in the sham group ( $8.8 \pm 6.2$ ) than that in the control group ( $P < 0.05$ , Fig. 1). These data suggested surgical wound could promote pulmonary metastasis of human breast cells.

**Surgical wound could enhance the interaction of cancer and host cells to produce Pro-MMP-9.** To evaluate the correlation between MMP-9 level in serum and surgical stress, we measured the postoperative levels of serum MMP-9 at the different time points by zymography. The host and human MMP-9 can be distinguished, as their molecular weights are apparently different [23]. As shown on Fig. 2, a, b, c, during days 1 to 3 after surgery, levels of host Pro-MMP-9 were significantly higher in the surgery and sham groups as compared with the control

group ( $P < 0.0001$ ), but no significant difference was observed between the surgery and sham groups. In the following two weeks three groups of mice showed levels of Pro-MMP-9 with mild difference. However, from day 19 the levels of host Pro-MMP-9 in the sham group became significantly higher than that in other two groups ( $P < 0.05$ ). The high levels in sham group were maintained to day 40. Notably, no activated MMP-9 was detected during whole process. In addition, the level of host Pro-MMP-9 in serum measured by ELISA at the day of 60 was similar to the level measured by zymography (data not shown), conforming the data were reliable. These observations not only indicated that surgical wound promoted expression of host Pro-MMP-9, but also implied that surgical wound resulted in interaction of cancer cells with host cells to produce Pro-MMP-9 during certain period after operation.



**Fig. 1.** Effects of surgical wound on the metastatic burden in lungs of nude mice with MDA-MB-435 primary tumor. *a*: Metastases on lung surface observed by microscopy ( $\times 8$ ). *b*: H&E-stained thin sections of lungs examined for the presence of metastases ( $\times 100$ ). *c*: The average metastatic burden in the lungs quantified using point counting method, (\*) for statistically significant when  $P < 0.05$ .



**Fig. 2.** Effects of surgical wound on the level of mouse Pro-MMP-9 in serum examined by zymography. *a*: The mouse Pro-MMP-9, instead of human pro-MMP-9, identified. Recombinant mouse Pro-MMP-9 standard was used as a marker. Abbreviations: M serum — mouse serum; H serum — human serum; Tumor — MDA-MB-435 primary tumor. *b*: The activity of mouse Pro-MMP9 at three time points. *c*: Quantitative analysis of Pro-MMP-9 activity determined by densitometry of respective bands (control group taken as 100%). The results shown in the histogram were the mean  $\pm$  standard deviation. As compared with control, (\*)  $P < 0.05$ , as compared with control and surgery, (\*\*)  $P < 0.05$ .

**Level of host Pro-MMP-9 correlated with tumor volume and the metastatic burden.** To evaluate the effect of host Pro-MMP-9 on growth and metastasis of breast cancer cells we examined tumor volume and metastatic burden in our animal model. As shown in Fig. 3, tumor volume in sham surgery group was larger during weeks 4 to 6, which corresponded to the postoperative period when higher serum Pro-MMP-9 level was observed. Statistic analysis indicated a positive correlation of host serum Pro-MMP-9 level and tumor volume ( $P < 0.05$ , Table 2). Furthermore, the level of host serum Pro-MMP-9 is positively correlated to metastatic burden ( $r = 0.52$ ,  $P = 0.0256$ ) at the endpoint of experiments.

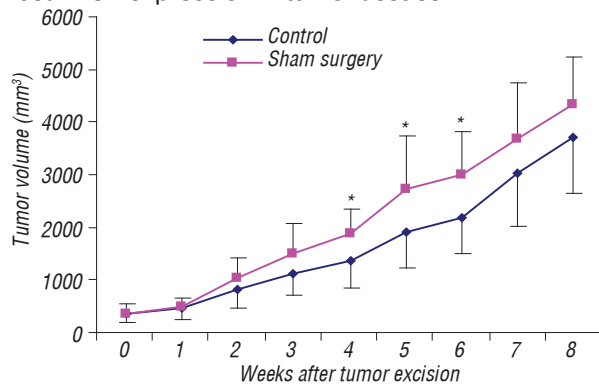
**Table 2.** Correlation between mouse Pro-MMP-9 level and tumor volume in sham surgical group.

Time (d)	19	26	33	40
Corr (r)	0.53	0.41	0.48	0.55
P value	0.011	0.042	0.014	0.015

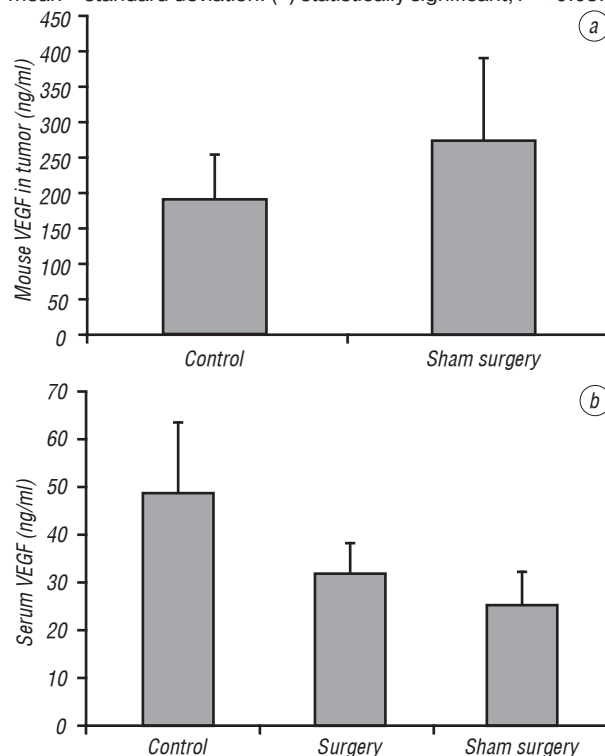
**Surgical wound promoted mouse VEGF expression in tumor.** To investigate the effect of surgical wound on the host VEGF expression, we measured the level of mouse VEGF in tumor by ELISA. Compared with control group, VEGF level of tumor tissues was



significantly higher in sham surgery group ( $P < 0.05$ , Fig. 4, a). The expression level of VEGF in blood serum was also measured. Interestingly, the level of serum VEGF was significantly lower in sham surgery group than in control group ( $P < 0.05$ , Fig. 4, b). These data suggested that surgical wound could mainly promote host VEGF expression in tumor tissues.



**Fig. 3.** Effects of surgical wound on the growth of MDA-MB-435 mammary primary tumors in nude mice. Tumor size was tested and tumor volume was calculated weekly. The results represent the mean  $\pm$  standard deviation. (\*) statistically significant,  $P < 0.05$ .



**Fig. 4.** Effects of surgical wound on mouse VEGF expression. a: ELISA validation of VEGF expression in tumor. b: ELISA validation of VEGF expression in serum. The results shown in the histogram were the mean  $\pm$  standard deviation. (\*) statistically significant ( $P < 0.05$ ) compared to control.

**DISCUSSION**

Our present study demonstrated surgical wound had a promoting effect on tumor growth and pulmonary metastasis of human breast cells, if tumor cells remain in bodies. This effect might be related to the postoperative interaction of cancer and host cells that resulted in production of host Pro-MMP-9. Surgical process could also increase the VEGF expression in tumor tissues. This implicated surgical wound-pro-

duced host Pro-MMP-9 and tumor cell VEGF might be important mediators leading to metastasis of residual breast cancer after surgery.

Recent studies have shown that the reactive stroma adjacent to carcinoma cells in wound repair is fundamentally different from the stroma of normal adult tissues in many human cancers [24]. Breast stroma accounts for more than 80% of the resting breast volume [25]. Interactions between cancer cells and surrounding stroma fibroblasts have been suggested to play a critical role in tumor invasion and metastasis [26–28]. Experimental *in vitro* and *in vivo* findings have indicated that interaction of tumor and stroma cells can mutually induce MMPs, which in turn can contribute to overall tumor invasion and metastasis [29–31]. The increased host serum Pro-MMP-9 and VEGF expression in tumor cells induced by operative wound might be resulted from the interaction of cancer cells and surrounding stromal cells. The highest level of host serum Pro-MMP-9 was observed between three to six weeks after operation. The increase level was positively related to enhanced tumor growth and metastasis, suggesting the level of serum Pro-MMP-9 in this time period would provide useful information for monitoring postoperative metastasis of breast cancer. Additionally, VEGF level in tumor tissues was informative with regard to the postoperative metastasis. Therefore, it deserves determining whether combination of serum Pro-MMP-9 and VEGF level in tumor tissues are sensitive markers to monitor tumor metastasis after operation in future. Our work implies that it is necessary to formulate more suitable treatment prescriptions to reduce the risk of tumor metastasis.

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## ХИРУРГИЧЕСКОЕ ВМЕШАТЕЛЬСТВО СТИМУЛИРУЕТ РОСТ ОПУХОЛИ И МЕТАСТАЗИРОВАНИЕ ЗА СЧЕТ АКТИВАЦИИ ЭКСПРЕССИИ MMP-9 И VEGF В МОДЕЛИ ОПУХОЛИ МОЛОЧНОЙ ЖЕЛЕЗЫ

Хирургическое лечение до сих пор остается главным терапевтическим подходом при лечении больных раком молочной железы. Хотя хирургическое удаление опухоли в большинстве случаев достаточно эффективно, в то же время оно может увеличить риск метастазирования остаточных опухолевых клеток. Понимание связи между метастазированием при раке молочной железы и операционной раной позволит выбрать наилучшую стратегию постоперационного лечения. *Цель:* определить влияние хирургического вмешательства на метастазирование опухоли. *Методы:* была создана модель рака молочной железы человека в виде ксенотрансплантата MDA-MB-435. Уровень Про-матричной металлопротеиназы 9 (Про-MMP-9) и фактора роста эндотелия сосудов (VEGF) в сыворотке реципиента и опухолях оценивались в разные временные промежутки методом ELISA и зимографии. При этом определяли наличие корреляции между этими показателями, ростом опухоли и постоперационными метастазами. *Результаты:* было показано, что операционная рана способствует росту опухоли молочной железы и ее метастазированию в легкие в случае, когда при удалении опухоли в ране остаются опухолевые клетки. Это может быть связано с постоперационным взаимодействием опухолевых и окружающих их нормальных клеток, которое приводит к экспрессии Про-MMP-9. Удаление опухоли может также способствовать увеличению экспрессии VEGF опухолевыми клетками. *Выводы:* Про-MMP-9, экспрессируемый нормальными клетками, окружающими постоперационную рану, а также VEGF, продуцируемый опухолевыми клетками, могут быть важными медиаторами метастазирования остаточных опухолевых клеток после хирургического вмешательства. *Ключевые слова:* операционная рана, метастазы, матричная металлопротеиназа 9, рак молочной железы, фактор роста эндотелия сосудов.