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Pre-treatment Mitotic Index Versus Computer-quantitated Ki-67 Nuclear Antigen Labeling Index as Predictors of Response to Neoadjuvant Chemotherapy in Uterine Cervical Carcinoma

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Abstract

In order to evaluate the usefulness of the mitotic index (MI) and the Ki-67 nuclear antigen labeling index (Ki67LI) in pre-treatment biopsy specimens as predictors of response to chemotherapy for uterine cervical carcinomas, twelve patients with squamous cell carcinoma who received neoadjuvant chemotherapy before radical surgery were investigated. The MI and computer-quantitated Ki67LI were determined using H&E and immunostained slides of biopsy specimens collected before chemotherapy. Tumor size was measured three-dimensionally by MR imaging, and assessed before and after chemotherapy. We compared the values of MI and Ki67LI with changes in tumor size and the following results were obtained.

1) The percentage reduction in tumor size ranged from 0 to 98%. The MI ranged from 0.5 to 15, and Ki67LI ranged from 0.01 to 50.1%. 2) A significant positive correlation was observed between response to chemotherapy assessed on MR image and MI [Spearman's correlation coefficient (r) = 0.66, n = 12, p = 0.027], and between response to chemotherapy and Ki67LI (r = 0.72, n = 12, p = 0.017). 3) A significant correlation was observed between MI and computer-assessed Ki67 LI [Pearson's correlation coefficient (r) = 0.80, n = 12, p = 0.002]. Therefore, pre-chemotherapy MI and Ki67LI were both good predictors of response to platinum-based chemotherapy. Because MI is technically more convenient and economically less expensive than computer-quantitated Ki67LI, MI remains a simple and reliable predictor from the clinical point of view. (J Nippon Med Sch 2003; 70: 219-226)

Key words: uterine cervical carcinoma, mitotic index, Ki-67 nuclear antigen labeling index, predictors of chemotherapy response, platinum regimen

Introduction

Although chemotherapy is one of the major treatment modalities for uterine malignancies, very few methods to predict response to various regimens have been reported. As *in vitro* methods, several chemosensitivity tests have been reported, including [³H]-uridine assay, MTT assay, colony forming assay, and adenosine triphosphate (ATP) chemosensitivity assays¹⁻⁵. However, no definitive *in vivo* indicator has been developed to predict response to chemotherapy for gynecologic malignancies. In the clinical setting, response to chemotherapy is usually evaluated by image diagnosis such as magnetic resonance imaging or pathological methods in which a percentage of necrosis is measured⁶⁻⁸. However, both methods can only evaluate how much a given tumor has responded to chemotherapy so far but do not predict how much the tumor will respond to chemotherapy in the future. Delineation of a predictor in pre-chemotherapy biopsies will be helpful to foretell future outcomes.

In a recent study with a limited number of cases, we showed that high MI and Ki67LI before chemotherapy and a marked decrease in MI shortly after chemotherapy appeared to predict good response to neoadjuvant chemotherapy in uterine cervical carcinomas⁹. We observed that it was important to follow the chronological changes of MI or Ki67LI in biopsies collected before and shortly after chemotherapy in order to predict chemotherapy response of the on-going regimen. Since considerable effort is needed to sample biopsies several times before and after chemotherapy, from the practical point of view, it is far more convenient if determination of a predictor in only one biopsy before chemotherapy can foretell the response to a particular regimen. Therefore, the purpose of this study was to examine whether pre-chemotherapy MI or Ki67LI can predict the response of uterine cervical squamous cell carcinomas to chemotherapy using cases treated with neoadjuvant chemotherapy.

Patients and Methods

Patients

Among 33 patients with uterine cervical carcinoma who received neoadjuvant chemotherapy before radical surgery or radiotherapy at the Department of Obstetrics and Gynecology, Nippon Medical School Main Hospital or Chiba Hokusoh Hospital between 1996 and 2001, 12 patients for whom all the necessary data were available were retrospectively studied. Informed consent had been obtained from the patients. The patients ranged in age from 34 to 60 years (mean 49 years). One patient was staged as II a, five as II b and six as IIIb following the classification of the International Federation of Gynecology and Obstetrics (FIGO). All twelve patients were diagnosed histologically as having invasive squamous cell carcinoma of the uterine cervix.

Neoadjuvant chemotherapy

Neoadjuvant chemotherapy was performed using the balloon-occluded arterial infusion (BOAI) method. A catheter was placed superselectively in each of the bilateral uterine arteries approached by the Seldinger method. Half of the total amount of chemotherapeutic agent was administered into each catheter. CDDP (75 mg/m² body surface area) was used as the main chemotherapeutic agent. During administration of the anticancer drug, the balloon was inflated so that arterial blood flow was interrupted, allowing the drug to be delivered into the lesion at a high concentration (i.e., without dilution by the blood flow).

Evaluation of chemotherapy response

MR imaging was performed with either an 0.5 T scanner (MRT-50A, Toshiba Medical Systems) or a 1.5 T scanner (Signa Horizon, GE Medical Systems, Milwaukee, WI, USA) with a body array coil. T2-weighted images were obtained in the planes along the long and short axes of the uterus and were reviewed by three observers (S.O., Y.A. and S.K.). The three dimensions of the tumor on images obtained before chemotherapy and 4 weeks after

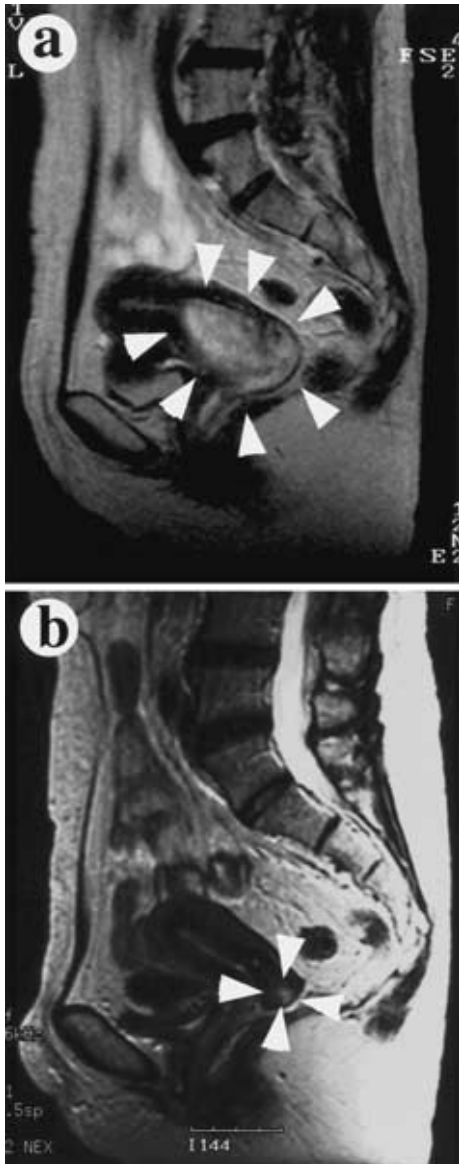


Fig. 1 Change of tumor size assessed on magnetic resonance (MR) images.

Case #1: The tumor is depicted as a high intensity area circumscribed by arrowheads on both MR images. Marked reduction of tumor size is recognized comparing MR images before chemotherapy (a) and 4 weeks after chemotherapy (b).

chemotherapy were measured, and consensus results of the three observers were obtained (**Fig. 1-a and b**). Tumor volume was calculated by length X width X depth. Reduction in tumor size was defined in terms of change in tumor volume: $100 \times (1 - \text{tumor volume after therapy} / \text{tumor volume before therapy})$.

Preparation of biopsy specimens

All specimens were 10% formalin-fixed and paraffin-embedded blocks of biopsies obtained before chemotherapy. Four- μm sections were prepared for hematoxylin and eosin (H&E) staining and immunohistochemistry. Ki67LI immunohistochemical staining was performed by the streptavidin-biotin methods using the DAKO LSAB kit (DAKO, Carpinteria, CA). Anti-MIB-1 mouse monoclonal antibody (diluted 1: 50; Immunotech, Marseille, France) was used as the primary antibody. For retrieval of antigenicity, specimens in 10 mM sodium citrate buffer (pH 6.0) were pretreated in a microwave oven at 95°C for 30 min. Sections from a tonsil with follicular hyperplasia served as positive controls. Negative control sections were prepared using non-immune γ -globulin in place of the primary antibody. All sections were counterstained with H&E and mounted in glycerin jelly.

Evaluation of MI

Sections stained with H&E were evaluated for MI under a microscope (Olympus BX50) with a 40X objective and a charge coupled device color camera (Sony DXC-107A; Tokyo, Japan) coupled to a monitoring system (Sony PVM-14450M) showing a field area of 0.1 mm \times 0.13 mm (2000-fold magnification). Multiple observers independently evaluated the same field on the monitor screen and a consensus on the final result was obtained.

Mitotic figures were identified using strict criteria. Only nuclei with definite morphologic features of metaphase, anaphase or telophase were counted. Hyperchromatic and apoptotic (karyorrhectic) nuclei were excluded. Karyolytic nuclei, which usually accompanied degenerated cytoplasm, were also excluded. Mitotic figures were counted in multiple randomly selected fields containing non-necrotic tumor components until the neoplastic cell count reached 1,000~3,000. The MI in this study was defined and expressed as the number of mitotic figures in 1,000 neoplastic cells (**Fig. 2-a and b**). These procedures were repeated three times and the final results were based on consensus among three observers (S.K., N.M. and Y.O.) who were blinded to the clinical information of the patients.

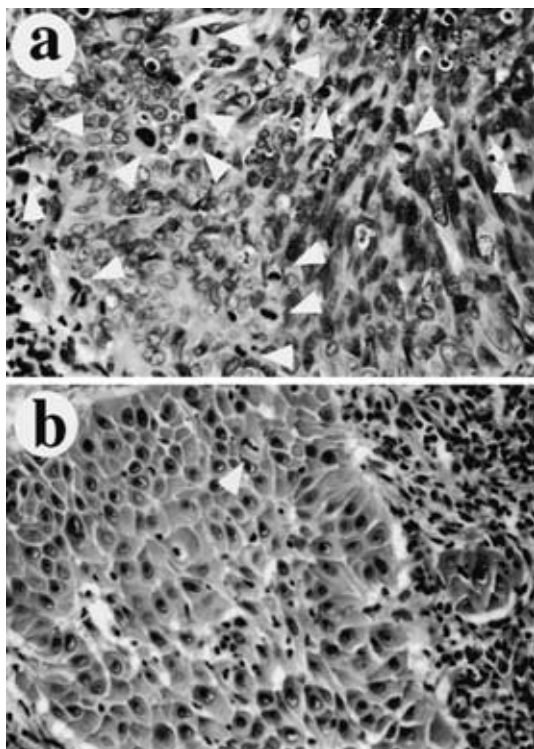


Fig. 2 Mitotic indices of biopsy samples collected before chemotherapy in cases showing the best (a) and least (b) response. White arrowheads show mitotic figures. Mitotic index before balloon-occluded arterial infusion (BOAI) is 15 in Case #1 (a) and 0.5 in Case #6 (b).

Evaluation of Ki67 LI

Using a modifying method of Amin et al., we evaluated Ki67LI by an automated method using the computer program Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, CA, USA) in conjunction with the public domain NIH Image (Version 1.61; Wayne Rasband, National Institute of Health, Bethesda, Md., USA).

For each case, sections immunostained with anti-MIB antibody for Ki67LI were scanned and three representative fields were photographed at a magnification of $50\times$ on 24×36 mm color film (PROVIA 100 F, FUJIFILM, Tokyo, Japan) using a photomicrography system (VANOX AHBS3, Olympus Optical Co., Ltd., Tokyo, Japan).

Each color slide image was converted into an RGB image file (680 pixels in width, 449 pixels in height, 300 pixels/inch in resolution) using a scanner (Polascan 35 Ultra, Polaroid Corp., Cambridge, MA,

USA) and Photoshop 5.0 running on a personal computer, Power Macintosh G4 (Apple Computer Inc, Cupertino, CA., U.S.A.) (Fig. 3-a). In each image, the stromal areas including vessels were painted white so that only tumor cells were counted (Fig. 3-b and g).

Each RGB image was converted to B and R images using the Photoshop (Fig. 3-c, d and h, i). The B image was produced by subtracting the blue component from the RGB image, and showed only brown MIB-1-positive nuclei (Fig. 3-h and i). The R image was made by subtracting the red component from the RGB image, and showed both blue (counterstained) MIB-1-negative nuclei and brown MIB-1-positive nuclei (Fig. 3-c and d). These R and B image data had to be saved into gray-scale form (8-bit grayscale, 300 pixels per inch, approximately 300 Kilobytes) to be processed by the NIH Image program (Version 1.61; Wayne Rasband, National Institute of Health, Bethesda, Md., USA).

For each imported B or R image, the threshold level was adjusted by using the "look-up table (LUT) tool". In the LUT window, only strong nuclear immunostaining was regarded as positive (Fig. 3-e, f and j, k). Then the minimum particle size was adjusted appropriately. The "Analyze Particle" command automatically counted the number of particles and the counting result was displayed in the "into" window (Fig. 3-l). Ki67LI was defined as the number of MIB-1-positive cells (cells counted in the B image) divided by the total number of cells (cells counted in the R image) in an area of 0.2834 mm^2 (%).

Statistical analysis

We compared MI values and Ki67LI before chemotherapy with tumor reduction rates assessed by MR image using Spearman's correlation coefficient. We also tested the correlation between MI and Ki67LI using Pearson's correlation coefficient by rank test. Statistical analyses were performed using the software StatView (SAS Institute Inc., Cary, NC, USA).

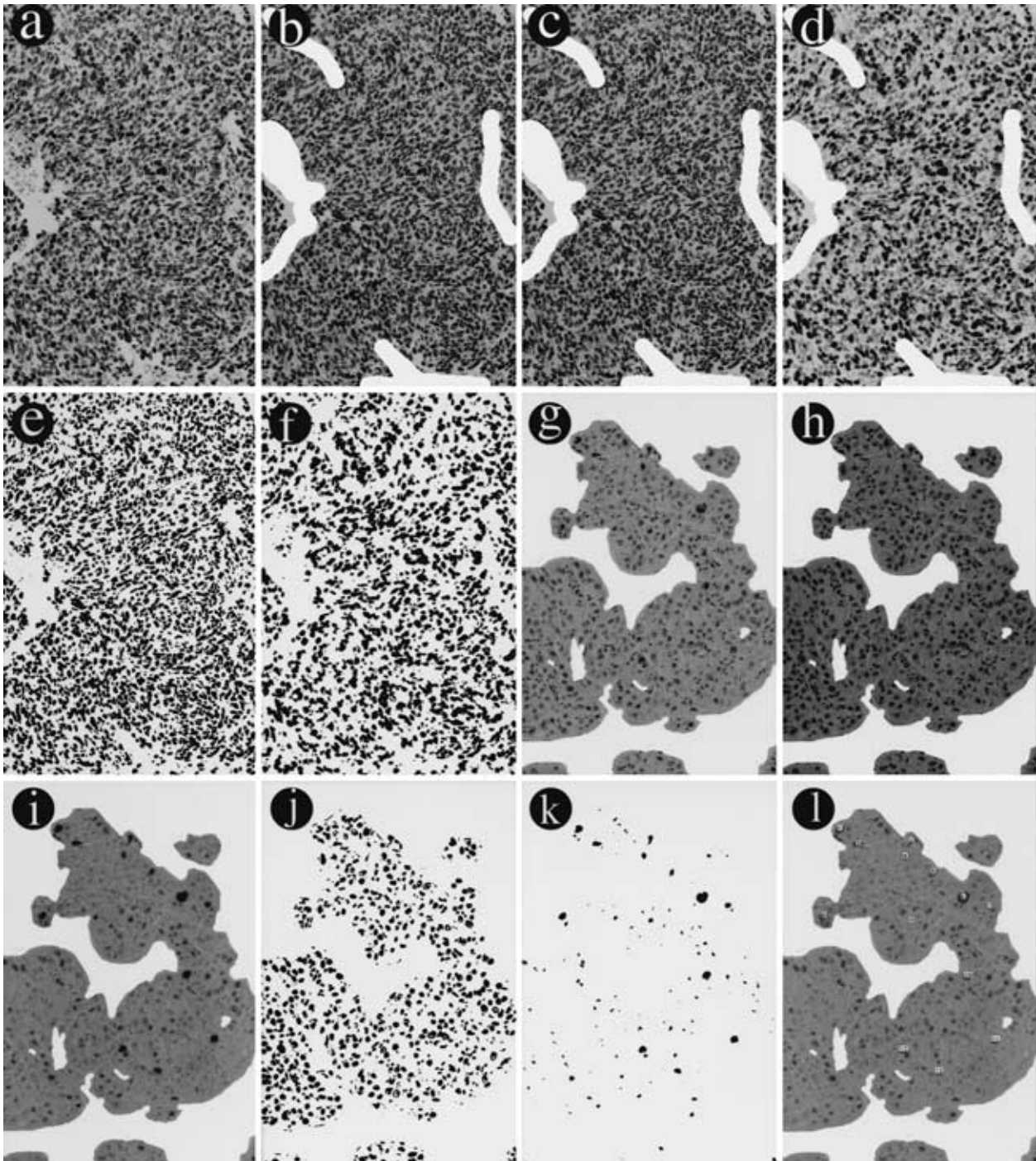


Fig. 3 Automated analysis of Ki 67 LI in cases showing the best (a to f) and least (g to l) chemotherapy responses

(a) A black and white photograph of RGB image for the MIB-1 immuno-stained slide in a case showing marked response. (b) A black and white photograph of the RGB image (a) in which stromal areas have been painted white to avoid counting of inflammatory and endothelial cells. (c) A gray scale expression of R channel image of the RGB image (b). The nuclei of both MIB-1-positive cells and -negative cells are recognized as darkly stained nuclei. (d) A gray scale expression of B channel image of the RGB image (b). Only the nuclei of MIB-1-positive cells are recognized as darkly stained nuclei. (e) In a LUT window of the NIH Image program, a converted image from the R-image shows both MIB-1-positive and -negative nuclei as multiple dark particles when the threshold level was adjusted. (f) In a LUT window of the NIH Image program, a converted image from the B-image shows only MIB-1-positive nuclei as multiple dark particles when the threshold level was adjusted. (g) A black and white photograph of the RGB image of a case showing weak response, in which the stromal areas have been painted white to eliminate counting of inflammatory cells and endothelial cells. (h) A gray scale expression of R channel image of the RGB image (g). The nuclei of both MIB-1-positive cells and -negative cells are recognized as darkly stained nuclei. (i) A gray scale expression of B channel image of the RGB image (g). Only the nuclei of MIB-1-positive cells are recognized as darkly stained nuclei. (j) In a LUT window of the NIH Image program, a converted image from the R-image showed both MIB-1-positive and -negative nuclei as multiple dark particles. (k) In a LUT window of the NIH Image program, a converted image from the B-image showed only MIB-1-positive nuclei as multiple dark particles. (l) Counting with the "Analyze Particle" command: If the least particle size has been adjusted properly, particles are counted automatically and the numbers of the counted nuclei are displayed instantly (In this case, a total of 13 nuclei have been counted) when the number is relatively small.

Results

The percentage reduction in tumor size of the 12 cases ranged from 0 to 98% (mean; 65.7%). MI of the 12 cases ranged from 0.5 to 15 (mean; 8.3). A positive and strong correlation was observed between MI and response to chemotherapy evaluated by MR imaging, as assessed by Spearman's correlation coefficient ($r=0.66$, $n=12$, $p=0.027$) (Fig. 4).

Therefore, MI determined in the biopsy specimen collected at the time of histological diagnosis might be a good predictor of response to future chemotherapy. Ki67LI determined using the computer program ranged from 0.01 to 50.1% (mean; 33.43%). A positive and strong correlation was also found between Ki67LI and chemotherapy response evaluated by MR imaging, as assessed by Spearman's correlation coefficient ($r=0.72$, $n=12$, $p=0.017$) (Fig. 4). Therefore, Ki67LI in the biopsy specimen may also be a good predictor of chemotherapy response. A positive and strong correlation existed between MI and Ki67LI, as assessed by Pearson's correlation coefficient ($r=0.80$, $n=12$, $p=0.002$) (Fig. 4).

Discussion

Several *in vitro* chemosensitivity tests to predict response to chemotherapy have been reported, but no *in vivo* index to predict clinical chemotherapeutic response has been delineated. For radiotherapy of cervical cancer, Ki67LI and MI values have been considered to be effective prognostic factors. According to Nakano et al.¹⁰, a higher MI value in the proliferating cell population in patients receiving radiotherapy is associated with a poor prognosis. However, no such index has been reported for chemotherapy. Therefore we assessed the usefulness of MI and Ki67LI determined in non-necrotic areas of the tumor as predictors of response to neoadjuvant chemotherapy for carcinomas of the uterine cervix.

Conventionally, the effect of chemotherapy is evaluated by assessing the area of necrosis in histological sections or changes in tumor size on MR

images^{6,11,12}. However, the change in tumor volume evaluated from MR images is not a predictor of the ongoing response to chemotherapy, as it merely indicates the result of chemotherapy at the time when the evaluation is made¹³. The situation is the same when using necrosis in the specimen resected at the time of radical surgery as an index. In contrast, necrotic changes observed in a biopsy specimen taken shortly after chemotherapy might be useful for prediction of the chemotherapy response. However, necrosis usually occurs so randomly that it is not possible to evaluate the overall damage by observing only the necrotic areas. Thus we have tried to find other changes that occur during the process of cellular damage.

In our study reported recently, we proposed that MI and Ki67LI estimated in the non-necrotic areas of biopsies collected before and after chemotherapy could be used as predictors of response to neoadjuvant chemotherapy for uterine cervical carcinomas⁹. The merit of this method is that it evaluates the change in biological activity of the tumor by comparing MI or Ki-67 nuclear antigen labeling index before and after chemotherapy. However this method has the demerit that serial biopsies have to be collected at various times.

In the present study, in order to avoid the elaborate efforts of biopsy collection, we proposed that only pre-chemotherapy MI or Ki67LI could be used to predict the outcome of future chemotherapy. Although Ki67LI has been recognized as a good indicator of cell proliferation, MI was a better predictor than Ki67LI in our previous study using a smaller number of cases. One of the reasons for this result was the technical problem associated with immunohistochemistry. In this study, we evaluated Ki67LI using a computer image analysis program.

In the past, two such methods have been reported^{14,15}. In both studies, NIH image program was applied to count MIB-1-positive cells and -negative cells automatically. However, the two studies differed in the method to identify MIB-1-positive cells and -negative cells. In the method by Ide et al.¹⁴, MIB-1-positive cells were marked with a white pen and MIB-1-negative cells with a black pen on the enlarged computer image. In this method,

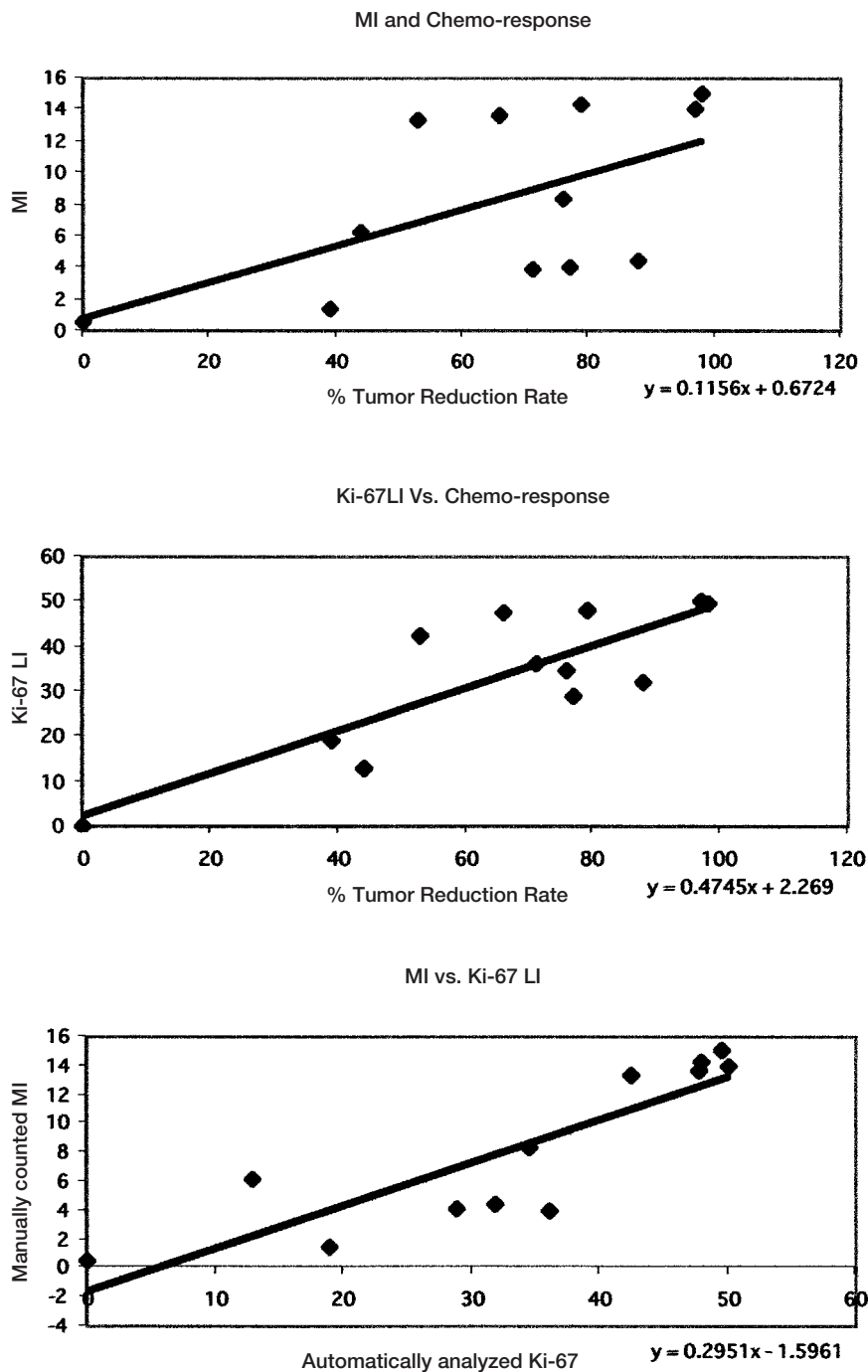


Fig. 4 A scattergram showing (upper) the response to chemotherapy assessed by MR image versus MI, (middle) the response to chemotherapy assessed by MR image versus Ki67LI, (bottom) MI by manual counting versus Ki67LI by automatic counting.

the NIH image recognized distinctly marked dots without miss-counting overlapping neighboring cells, hematogenous cells, or endothelial cells. In this method, judgement of positivity was a subjective process, as is common in pathological examinations.

On the other hand, in the method of Amin et al.¹⁴, MIB-1-positive cells and -negative cells were judged

automatically using the NIH Image software. In this process, the judgement of positivity was performed objectively by proper adjustment of standards. However, their method could not avoid counting endothelial cells and some inflammatory cells as MIB-1-negative cells, and some inflammatory cells as MIB-1-positive cells, as well as indiscriminately

counting neighboring or overlapping cells.

In our present study, we used the latter method to evaluate Ki67LI by masking the stromal, vascular and inflammatory cell-rich sites using white paint. The result of this method appears fairly good as shown by a good correlation between MI and Ki67LI and also a strong correlation between Ki67LI and tumor response to chemotherapy. Therefore, we consider Ki67LI determination by automatic counting using the NIH Image program to be a convenient and reliable method. However, while Ki67LI demonstrates almost the same ability as MI to predict the future chemotherapy response, determination of Ki67LI requires a more complex process and equipment. Therefore, we conclude that MI is a simple and good predictor of response to chemotherapy using cis-platinum based combined regimen.

In summary, from the study of 12 cases of uterine cervical carcinoma treated with neoadjuvant chemotherapy, we proved the usefulness of manual determination of MI in a pre-treatment biopsy specimen as a predictor of chemotherapy response compared to automated computer analysis of Ki 67 KL.

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References

1. Ford CH, Richardson VJ, Tsaltas G: Comparison of tetrazolium colorimetric and [³H]-uridine assays for in vitro chemosensitivity testing. *Cancer Chemother Pharmacol* 1989; 24: 295-301.
2. Fanning J, Biddle WC, Goldrosen M, et al: Comparison of cisplatin and carboplatin cytotoxicity in human ovarian cancer cell lines using the MTT assay. *Gynecol Oncol* 1990; 39: 119-122.
3. Umbach GE, Poethen J, Matthiessen H von, Bender HG, Koldovsky U: Experiences with the human tumor colony-forming assay in gynecologic malignancies. *J Cancer Res Clin Oncol* 1985; 110: 234-237.
4. Untch M, Sevin BU, Perras JP, et al: Chemosensitivity to the new anthracycline pirarubicin and other chemotherapeutic agents in primary and recurrent ovarian tumors in vitro. *Gynecol Oncol* 1992; 47: 172-178.
5. Sevin BU, Perras JP, Averette HE, Donato DM, Penalver M: Chemosensitivity testing in ovarian cancer. *Cancer* 1993; 71: 1613-1620.
6. Van der Woude HJ, Bloem JL, Taminiou AH, Nooy MA, Hogendoorn PC: Classification of histopathologic changes following chemotherapy in Ewing's sarcoma of bone. *Skeletal Radiology* 1994; 23: 501-507.
7. Manfredi R, Maresca G, Smaniotto D, et al: Cervical cancer response to neoadjuvant therapy: MR imaging assessment. *Radiology* 1998; 209: 819-824.
8. Gels, ME, Nijboer AP, Hoekstra HJ, et al: Complications of the post-chemotherapy resection of retroperitoneal residual tumour mass in patients with non-seminomatous testicular germ cell tumours. *British Journal of Urology* 1997; 79: 263-268.
9. Kamoi S, Ohaki Y, Okada S, et al: Mitotic Index and Ki-67 Nuclear Antigen Labeling Index as Predictors of Chemotherapy Response in Uterine Cervical Carcinoma. *Gynecol Oncol* 2001; 83: 555-559.
10. Nakano T, Oka K: Differential values of Ki-67 index and mitotic index of proliferating cell population. An assessment of cell cycle and prognosis in radiation therapy for cervical cancer. *Cancer* 1993; 72: 2401-2408.
11. Bacci G, Picci P, Avella M, et al: Effect of intra-arterial versus intravenous cisplatin in addition to systemic adriamycin and high-dose methotrexate on histologic tumor response of osteosarcoma of the extremities. *Journal of Chemotherapy* 1992; 4: 189-195.
12. Bacci G, Ferrari S, Forni C, et al: The effect of intra-arterial versus intravenous cisplatin in the neoadjuvant treatment of osteosarcoma of the limbs: the experience at the Rizzoli Institute. *Chirurgia Degli Organi di Movimento* 1996; 81: 369-382.
13. Abudu A, Davies AM, Pynsent PB, et al: Tumour volume as a predictor of necrosis after chemotherapy in Ewing's sarcoma. *Journal of Bone & Joint Surgery-British Volume* 1999; 81: 317-322.
14. Ide M, Jimbo M, Yamamoto M, Kubo O: Tumor Cell Counting Using an Image Analysis Program for MIB-1 Immunohistochemistry. *Neurol Med Chir* 1997; 37: 157-162.
15. Amin MR, Kurosaki M, Watanabe T, Tanaka S, Hori T: A comparative study of MIB-1 staining indices of gliomas measured by NIH Image analysis program and conventional manual cell counting method. *Neurol Res* 2000; 22: 495-500.

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