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# Changes in Cell Proliferative Parameters of SCCVII and EMT6 Murine Tumors after Single-dose Irradiation

Rumi Murata,[1,4] Yasumasa Nishimura,[1] Yuta Shibamoto,[2] Masahiro Hiraoka[1] and Mitsuyuki Abe[3]

*[1]Department of Radiology, Faculty of Medicine, [2]Department of Oncology, Chest Disease Research Institute, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-01 and [3]Kyoto National Hospital, 1-1 Mukaihata-cho, Fukakusa, Fushimi-ku, Kyoto 612*

**To understand better the repopulation kinetics of tumor cells after radiotherapy, we investigated changes in cell proliferative parameters after single-dose irradiation of SCCVII tumors in C3H/He mice and EMT6 tumors in Balb/c mice. The following parameters were determined 0–15 days after single irradiation at 20 or 30 Gy; dividing fraction (DF), potential doubling time (Tpot), number of clonogenic cells per tumor (Ncln), and volume doubling time (Tvol). DF and Tpot were determined by *in vivo-in vitro* cytokinesis-block assay with cytochalasin B, Ncln was measured by *in vivo-in vitro* colony-forming assay, and Tvol was determined by growth delay assay. In both tumors, longer Tpot and lower DF and Ncln were obtained for 3–4 days after irradiation, but in SCCVII tumors these values returned to the pretreatment levels 9 days after irradiation. In EMT6 tumors, Tpot, DF, and Ncln did not return to the pretreatment levels even 12 days after irradiation. In the regrowth phase of both tumors following irradiation at 20 Gy, Tvol was longer than the pretreatment level, although Tpot was similar in SCCVII and only slightly longer in EMT6. Therefore, the cell loss factor in the regrowth phase was considered to be higher than the pretreatment level in both tumors. From the results, recruitment of previously quiescent cells into the proliferative pool in these tumors was suggested to contribute to repopulation after radiation.**

Key words: Cell kinetics — Repopulation — Radiation — Murine tumor — Potential doubling time

Repopulation of tumor cells occurring during the course of fractionated irradiation is considered as one of the major causes of radiation treatment failure. One of the cell kinetic parameters, potential doubling time (Tpot) before treatment has been regarded as a good indicator of repopulation of surviving cells,[1,2] because tumors with a short pretreatment Tpot may repopulate rapidly during fractionated radiotherapy.[3–5] Some clinical studies have demonstrated that patients whose tumors had a shorter Tpot benefited from an accelerated radiation therapy schedule compared with a conventional schedule.[5–7] Therefore, pretreatment measurement of Tpot might be helpful in planning optimal treatment. However, the relationship between the pretreatment Tpot and the actual repopulation during or after treatments is still unclear.[5,8] In some murine tumors and a xenografted human sarcoma, it was observed that the effective doubling time of clonogens (Teff) during fractionated radiotherapy became shorter than the pretreatment Tpot,[8–10] indicating that accelerated repopulation occurred during fractionated radiotherapy. The repopulation rate may not be constant throughout the course of radiotherapy.[5] Tpot or some other cell kinetic parameters may change after a single dose of irradiation or during fractionated irradiation.

To understand the changes of tumor cell kinetics after radiotherapy, many experimental studies on tumor repopulation occurring after single-dose irradiation or during fractionated irradiation have been performed.[8,9,11–21] Changes in some kinetic parameters including Tpot, clonogenic fraction and dividing fraction have been demonstrated.[8,9,13,14,18–21] However, in most studies, only one or two of the parameters were measured at one arbitrary time point to analyze the proliferation of tumor cells. In addition, the results were not consistent even after a single dose of irradiation.[18,19,21] The phenomena occurring in tumors after irradiation seem to be too complicated to explain in terms of one or two kinetic parameters. Therefore, in the present study, five cell kinetic parameters, Tpot, dividing fraction (DF), number of clonogenic cells per tumor (Ncln), volume doubling time (Tvol), and cell loss factor (CLF), were estimated.

Cytochalasin B inhibits cytokinesis but preserves nuclear division, so cells undergoing mitoses are easily recognized by their multinucleate appearance. The *in vivo-in vitro* cytokinesis-block assay with cytochalasin B has enabled us to determine proliferative activity of

[4] To whom all correspondence should be addressed.

tumors.[22,23] Cell kinetic parameters, DF and Tpot could be estimated from the percentage of multinucleate cells, and the number of nuclei per cell, respectively.[23] Thus, DF and Tpot were determined easily and independently by this method. In previous studies, DF and Tpot determined by using the cytokinesis-block assay with cytochalasin B were found to correlate well with labelling index and Tpot obtained by a bromodeoxyuridine flow cytometry method.[23,24] In addition, the cytokinesis-block assay with cytochalasin B can be applied to human tumors to reveal changes in cell kinetics following radiotherapy.[24] Therefore, we used this assay to estimate DF and Tpot.

## MATERIALS AND METHODS

**Animals and tumors** SCCVII tumors in C3H/He mice and EMT6 tumors in Balb/c mice were used.[25,26] Mice were all male, 8 weeks of age at the start of experiments, and were kept in our microorganism-free animal facility. Shortly before the experiments, the cells were thawed from the original stocks and cultured in Eagle's minimum essential medium (MEM) supplemented with 12.5% fetal bovine serum (FBS) under humidified conditions at 37°C with 5% $CO_2$. Exponentially growing cells ($2\times10^5$) from monolayer cultures were inoculated subcutaneously into the right thigh of each mouse. Irradiation was started when the tumors reached approximately 500 $mm^3$.

**Irradiation** Before irradiation, mice were anesthetized by intraperitoneal administration of pentobarbital (60 mg/kg). To unify the pretreatment $O_2$ level of tumor cells, the root of the right thigh was ligated with string and radiation was given under hypoxic conditions. Mice were fixed in a jig with adhesive tape under anesthesia. Irradiation was started 5 min after the ligation and given locally to tumors with 10 MV X-rays generated by a linear accelerator at a dose rate of approximately 5.0 Gy/min. The field size was 3 × 30 cm. Radiation dose was 20 or 30 Gy for SCCVII tumors and 20 Gy for EMT6 tumors.

***In vivo-in vitro* colony formation assay** In *in vivo-in vitro* assay, tumors were excised, minced with scissors, and stirred with 0.1% neutral protease for 40 min at 37°C to prepare single cell suspensions. Then, appropriate numbers of viable tumor cells were plated on 60 mm tissue culture dishes, cultured in Eagle's MEM supplemented with 12.5% FBS under humidified conditions at 37°C with 5% $CO_2$, and 10 days later, colonies were fixed with ethanol and stained with Giemsa. The fraction of cells surviving (SF) and the number of viable cells obtained from one tumor (Ncell) were determined and Ncln values were calculated as follows; Ncln = SF × Ncell.

***In vivo-in vitro* cytokinesis-block assay with cytochalasin B** This assay was performed at the same time as the colony formation assay. From a single cell suspension, approximately $1\times10^5$ cells were plated on 60 mm tissue culture dishes and cultured with 2 μg/ml of cytochalasin B (Sigma Chemie, Deisenhofen, Germany) dissolved in dimethyl sulfoxide. Cultures were terminated at various intervals between 24 and 96 h. The cells were fixed with 3 : 1 ethanol/acetic acid, then stained with propidium iodide. In the presence of cytochalasin B, the number of nuclei increases while the cell number remains constant. The cells with different numbers of nuclei were counted

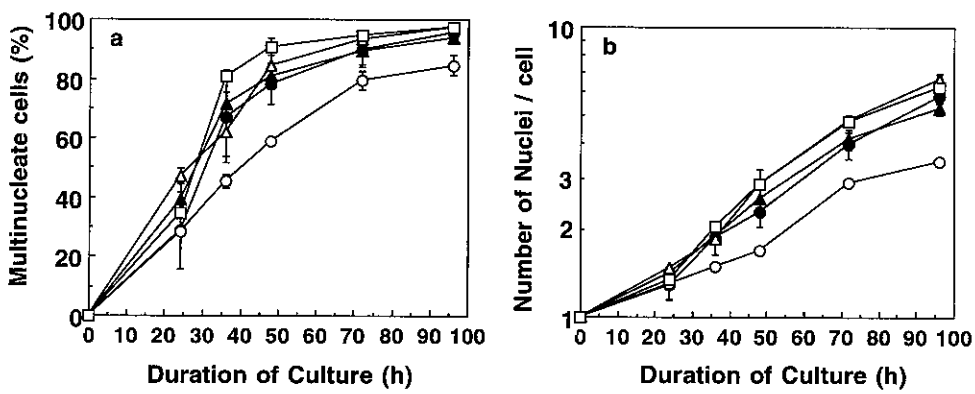


Fig. 1. a, The percentage of multinucleate SCCVII tumor cells is shown versus the incubation time. Cells were cultured in the presence of cytochalasin B. Tumor excision assay was carried out 3 (○), 6 (▲), 9 (△) or 15 days (□) after irradiation at 20 Gy. The data for control tumors are indicated with closed circles (●). Each point shows the mean value determined from 6 tumors and vertical bars represent standard errors of the mean. The dividing fraction was defined as the percentage of multinucleate cells at 72 h. b, The average number of nuclei per SCCVII tumor cell is plotted against the incubation time. The assay performed was the same as that in Fig. 1a. The potential doubling time was defined as time taken for the average number of nuclei to double.

under a fluorescence microscope, and the percentage of multinucleate cells (MNC) and the average number of nuclei per cell (N/C) were calculated. Fig. 1 shows an example of changes in MNC (%) and N/C. The percentage of MNC is considered to be the fraction of tumor cells undergoing mitosis. DF was defined as the percentage of MNC at 72 h, because it reached a plateau at that time (Fig. 1a).[23] Tpot is a hypothetical time required for the tumor cell population to double in number, if the population were not decreased by natural cell loss. In the cytokinesis block assay, Tpot was defined as the time for N/C to double (Fig. 1b). In the previous study, 2 $\mu$g/ml of cytochalasin B did not affect the dividing speed of nuclei.[23]

**Tumor growth delay assay** The tumor diameters were measured every other day with calipers. The tumor volume was determined by use of the formula $\pi/6 \times a \times b^2$, where $a$ is the longer and $b$ the perpendicular shorter tumor axis. Eight to ten mice were used for each treatment group. Tvol was defined as the time for a tumor to double in volume from 700–800 mm$^3$. For irradiated tumors, Tvol was determined in the regrowth phase. Tvol was measured for each tumor, and the median Tvol and its 95% confidence limits were calculated by logit analysis.[27] CLF in the regrowth phase was estimated using the formula,[28] CLF = 1 − (Tpot/Tvol). Student's $t$ test was used for statistical analysis.

## RESULTS

Growth curves for SCCVII and EMT6 tumors are shown in Fig. 2. In the regrowth phase after 20 or 30 Gy irradiation, exponential growth was observed for both tumors. The growth curves in the regrowth phase after irradiation were not as steep as those for control tumors, indicating that Tvol was prolonged in the regrowth phase for both tumors.

Changes in DF after irradiation at 20 or 30 Gy are shown in Fig. 3a. DF for untreated EMT6 or SCCVII tumors was 93±3% (mean±SE) or 90±5%, respectively. In SCCVII tumors, DF continued to decrease until 8 days after irradiation at 30 Gy, whereas DF began to increase 3 days after irradiation at 20 Gy and returned to the control level 6 days after treatment. In EMT6 tumors, DF temporarily decreased and started to increase 6 days after irradiation. However, DF did not return to the preirradiated level even 12 days after irradiation. Fig. 3b demonstrates changes in Tpot after a single fraction of 20 or 30 Gy. Pretreatment Tpot values were 47±2 h (mean±SE) for EMT6 tumors and 38±3 h for SCCVII tumors. For both tumors, Tpot was temporarily prolonged after irradiation. In EMT6 and SCCVII tumors receiving 20 Gy, Tpot values started to recover toward the preirradiated level 3–4 days after irradiation. In EMT6 tumors, Tpot had not returned to the preirradiated level even 12 days after irradiation. On the other hand, in SCCVII tumors, Tpot became shorter than the pretreatment Tpot 12 days after irradiation, although the difference was not significant. The prolongation of Tpot was more apparent after irradiation at 30 Gy than after 20 Gy.

Changes in Ncln are plotted as a function of time after irradiation in Fig. 4. Ncln decreased immediately after irradiation and started to recover 3–4 days after irradiation at 20 Gy. In EMT6 tumors, Ncln had not returned to the untreated level even 12 days after irradiation. When 20 Gy was given to SCCVII tumors, the recovery of Ncln was apparent 3 days after irradiation.

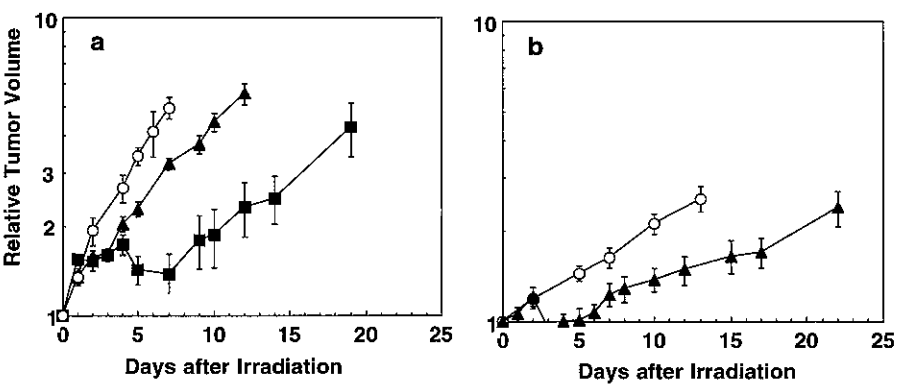


Fig. 2. a, Growth curves for SCCVII tumors, unirradiated (○) and irradiated with 20 Gy (▲) or 30 Gy (■). Each point shows the mean value determined from 8–10 tumors and vertical bars represent standard errors of the mean. b, Growth curves for EMT6 tumors, unirradiated (○) or irradiated with 20 Gy (▲). Each point shows the mean value determined from 8–10 tumors and vertical bars represent standard errors of the mean.

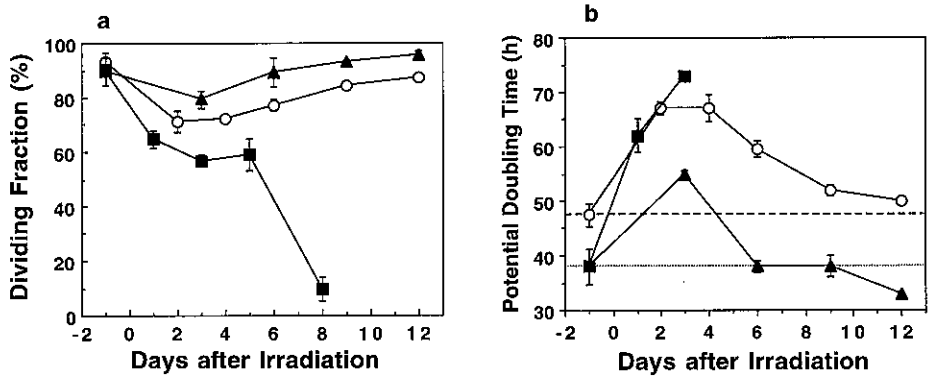


Fig. 3. a, Changes in dividing fraction (DF) as a function of time in EMT6 tumors irradiated with 20 Gy (○) and SCCVII tumors irradiated with 20 Gy (▲) or 30 Gy (■). Values of DF were determined from Fig. 2a. DF values for control tumors are shown on Day-1. Each point was determined from 6 tumors and vertical bars represent standard errors of the mean. b, Changes in potential doubling time (Tpot) as a function of time in EMT6 tumors irradiated with 20 Gy (○) and SCCVII tumors irradiated with 20 Gy (▲) or 30 Gy (■). Values of Tpot were determined from Fig. 2b. Tpot values for control tumors are shown on Day-1. The upper horizontal dotted line shows the pretreated Tpot value of EMT6 tumors, while the lower line shows that of SCCVII tumors. Each point indicates the mean value from 6 tumors and vertical bars represent standard errors of the mean.

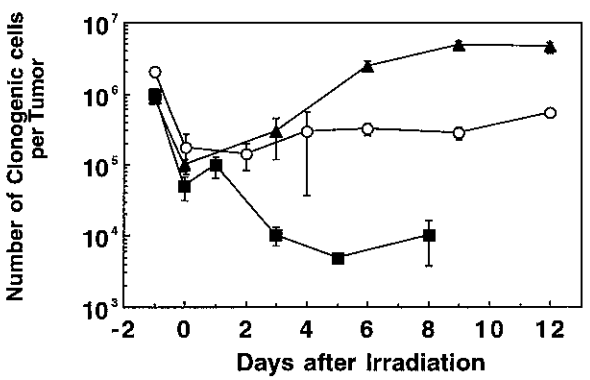


Fig. 4. Changes in the number of clonogenic cells per one tumor (Ncln) with time in EMT6 tumors irradiated with 20 Gy (○) and SCCVII tumors irradiated with 20 Gy (▲) or 30 Gy (■). Values of Ncln were determined by colony formation assay. The values for pretreated tumors are shown on Day-1. Each point shows the mean value from 6 tumors and vertical bars represent standard errors of the mean.

Tvol, Tpot, DF and CLF of control tumors and in the regrowth phase of irradiated tumors are shown in Table I. Tvol values in the regrowth phase increased significantly compared with those of control tumors ($P<0.001$, for both tumors). On the other hand, changes in Tpot in the regrowth phase were not substantial. Consequently, CLF calculated based on Tvol and Tpot increased significantly in the regrowth phase for both tumors compared with control tumors ($P<0.01$). DF in the regrowth phase of SCCVII tumors was increased slightly, while that of EMT6 tumors was decreased compared with those of preirradiation tumors.

## DISCUSSION

Pretreatment Tpot has been expected to be useful for predicting the repopulation rate of surviving cells during fractionated radiotherapy. However, the relationship between the pretreatment Tpot and Teff during fractionated irradiation is not clear, because the repopulation rate can change throughout the course of treatment.[5] Allam *et al.*[10] found shortening of Teff from pretreatment Tpot of 2.38 days to 1.35 days in a xenografted human sarcoma during a treatment schedule delivered over 30 days. Begg *et al.*[9] also reported shorter Teff compared with pretreatment Tpot in two murine squamous cell carcinomas. On the other hand, Speke and Hill[8] found that four of five tumors showed prolongation of Teff compared with pretreatment Tpot, while only SCCVII tumor showed shorter Teff compared with pretreatment Tpot. Therefore, pretreatment measurements of Tpot may not always predict Teff during fractionated radiotherapy. Although Tpot has been regarded as one of the most useful proliferation parameters for studies of tumor repopulation, changes in the other cell kinetic parameters such as DF, cell cycle time and CLF can also affect tumor repopulation during or after radiotherapy. However, to our knowledge, only a few studies have measured these proliferative parameters even after a single dose of irradiation. In this study, changes in five

Table I. Cell Kinetic Parameters in the Regrowth Phase Following Irradiation for SCCVII and EMT6 Tumors

| | SCCVII | | | EMT6 | |
|---|---|---|---|---|---|
| | control | 20 Gy | 30 Gy | control | 20 Gy |
| Tvol[a)] | 2.7 (2.5–2.9) | 4.1 (3.7–4.5) | 5.2 (4.4–6.1) | 7.1 (6.6–7.7) | 11.5 (10.7–12.3) |
| Tpot[b)] | 38±3 (h) | 38±2 (h) | | 47±2 (h) | 50±1 (h) |
| DF[c)] | 90±5 (%) | 93±0.4 (%) | | 94±3 (%) | 87±0.5 (%) |
| CLF[d)] | 41±5 (%) | 61±2 (%) | | 72±1 (%) | 82±1 (%) |

*a*) Tvol: Volume doubling time (days). Tvol was defined as the time for tumor volume to double from 700–800 mm$^3$. The value is shown as the median value from 8–10 tumors and 95% confidence limits of the value.
*b*) Tpot: Potential doubling time. The value was determined from Fig. 3b at the median time used for the determination of Tvol. The value is shown as the mean and standard error.
*c*) DF: Dividing fraction. The value was determined from Fig. 3a at the median time used for the determination of Tvol. The value is shown as the mean and standard error.
*d*) CLF: Cell loss factor. The value was calculated by use of the formula; CLF=1−(Tpot/Tvol) (ref. 28). The value is shown as the mean and standard error.

parameters, Tpot, DF, Ncln, Tvol, and CLF, after a single dose of irradiation were investigated using two murine tumors.

In the present study, prolongation of Tpot and decrease of DF and Ncln were temporarily observed after irradiation of both SCCVII and EMT6 tumors. After 20 Gy irradiation, the changes in tumor proliferative parameters including Tpot, DF and Ncln correlated well with each other. Within a few days after irradiation, tumor cells inactivated by irradiation might retain morphological characteristics similar to those of surviving tumor cells, and this may influence the determination of DF and Tpot. However, a post treatment lag period before the onset of repopulation of tumor cells appears to be a general feature of rodent tumors irradiated with high single X-ray doses.[14, 18, 20] The lag time before the tumor repopulation has been explained in terms of radiation-induced cell division delay.[12] The cell division delay may also be one of the causes of the prolongation of Tpot in the present study. Proliferating cells have been reported to have higher radiosensitivity than quiescent cells.[29] Therefore, immediately after irradiation, the proportion of proliferating cells may be decreased. Increase in the proportion of quiescent cells was suggested by temporary decrease in DF in this study. The recovery of DF observed in this study might be partly due to recruitment of quiescent cells into the proliferating state.

Changes of proliferative parameters depended on radiation dose. For SCCVII tumors, decrease in DF and Ncln and prolongation of Tpot were more prominent after 30 Gy of irradiation than after 20 Gy. Determination of DF, Tpot and Ncln was terminated before the onset of repopulation for SCCVII tumors receiving 30 Gy, because *in vivo-in vitro* assay tends to be inaccurate when the survival rate and proliferative activity of clonogens are very low. As tumor regrowth after irradiation at 30 Gy can be seen in Fig. 2a, recovery in Tpot, DF and Ncln might have been observed if they had been followed for a longer period. Changes of proliferative parameters are also influenced by the tumor type. For SCCVII tumors, DF, Tpot and Ncln returned to the pretreatment level by 6 days after 20 Gy irradiation and exceeded the pretreatment level thereafter, while in EMT6 tumors the parameters had not returned to the pretreatment level even 12 days after irradiation. In the present study, no significant increase in proliferative parameters was observed compared with the pretreatment levels after a single dose of irradiation for either of the tumors, although CLF appeared to increase significantly after irradiation. This indicates that accelerated repopulation did not occur. The experimental murine tumors used in this study originally had high proliferative activity. It might be one reason for the absence of accelerated repopulation.

Three mechanisms have been proposed for accelerated tumor repopulation: decrease in cell loss factor, recruitment of previously quiescent cells into the proliferative pool, and shortening of the cell cycle time of clonogenic cells.[19] Ang *et al.*[19] assessed changes in the cell-cycle time of clonogens of a murine fibrosarcoma and concluded that repopulation might not be due to shortening of the cell-cycle time. Szczepanski and Trott[21] also observed prolongation of the cell-cycle time after irradiation in a murine adenocarcinoma. However, Hermens and Barendsen[18] reported shortening of the cell-cycle time in a rat rhabdomyosarcoma. Thus, the results concerning changes of cell-cycle time after irradiation have not been consistent. On the other hand, mathematical

modelling predicts that accelerated repopulation requires a decrease in CLF.[30] Speke and Hill[8] suggested that the rapid repopulation in SCCVII tumors might be due to a change in growth fraction or a reduction in cell loss rate. However, CLF values before and after irradiation have rarely been determined. Hermens and Barendsen[18] found CLF at the fourth day after a single dose of 30 Gy was much larger than that of nonirradiated tumors. We observed increase of CLF in the regrowth phase after irradiation. One explanation for the increase of CLF might be the tumor bed effect.[31, 32] Radiation causes vascular damage which leads to deprivation of oxygen and nutrition from tumor cells. As a result, loss of tumor cells may increase. Therefore, decrease in CLF may not always contribute to tumor repopulation after irradiation. Consequently, recruitment of previously quiescent cells into the proliferative pool may contribute to tumor repopulation following irradiation in some tumors. This might be supported by our observation that DF began to increase after a temporary reduction.

In conclusion, five cell kinetic parameters, Tpot, DF, Ncln, Tvol and CLF, changed with time after a single dose of irradiation. Changes in Tpot, DF, and Ncln correlated well with each other. The two murine tumors showed elongation of Tvol at the regrowth phase, but this was not explained by the changes in Tpot. The CLF was considered to increase after irradiation in both tumors. Recruitment of previously quiescent cells into the proliferative pool was considered to contribute to tumor cell repopulation in these tumors.

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