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Existence and Significance of Non-Autonomous Cancer Replication for Clinical Management of Cancer: Observations and Hints from Ovarian Cancer Cases

Kangla Tsung*, Zhang Xu and Zhang Hui

Research Institute for Clinical Management of Cancer, Beijing, China

ABSTRACT

Cancer cells progress through replication. What has been puzzling to cancer surgeons and oncologists is not why cancer cells continue to grow, but why some of them do not grow steadily as expected and this behavior varies between cases and even between lesions in the same case. Our previous reports have addressed the issue of tight control of tumor progression by targeting only a minority part of cells in a tumor by targeted drugs such as TK inhibitors in lung cancer. The reconciliation of this situation brought out the concepts of autonomous and non-autonomous tumor replication that divide the mode of cancer cell replication into two categories based on the source of molecular signals that drive tumor replication. This establishment of the mode of tumor replication is very useful for clinical management of cancer, especially critical for risk evaluation of post-surgery cancer recurrence in almost every case. In this report, we apply this concept in the analyses and management of two ovarian cancer cases to show that the mode of tumor replication is a new dimension in cancer staging on top of the current TNM staging system and aids individualized case assessment and management.

***Corresponding author**

Kangla Tsung, Research Institute for Clinical Management of Cancer, Beijing, China; Email: tansuodexin@tanlunforcancer.net

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Introduction

In 1950 J. Engelbert Dunph, a young surgeon from Harvard Medical School, published an essay in *The New England Journal of Medicine* in which he described 4 cancer cases to illustrate that cancer is not a disease of steady and irrevocable growth as the mainstream had expected, but a disease with unpredictable behavior and outcome [1]. The question to be answered, as in his words, “is not what makes the cells suddenly grow, but what has held them in abeyance for long”. With this question answered, he hoped in the future, “it may be possible to determine the growth curves of a given tumor so as to plan extirpation at periods of quiescence rather than during periods of very active growth”. Dr. Dunphy had subsequently warned: “Until an accurate appraisal of the growth potentialities of any given tumor can be made, the surgeon must continue to grope in comparative darkness” [2]. Seventy years has passed since Dunphy raised the cancer puzzle, yet we still know very little about the growth propensities in each cancer cases to select the best treatment strategy for that case. On the one hand, we already know quite a lot of tumor biology as today’s genetic testing can often pinpoint the reason for cancerous growth in many tumors (oncogenes and replication-driven mutations in them). On the other hand, even with the success of drugs targeting tumor replication accurately, we have not explained some discrepancy between our knowledge and the clinical reality. For example, in our recent report we have addressed the observation that most tight antitumor control by targeted therapy drug (such as TK inhibitors) can only act on a minority part of tumor cells in a tumor as genetic testing has clearly shown that not all of the tumor cells in a tumor tissue contain the targeted mutation (a concept called

“mutation frequency”) [3]. Then what are those other non-target tumor cells and why don’t they progress under TKI therapy? The result of the exploration into this seemingly paradoxical observation has led to the discovery of two modes of tumor replication: the autonomous or self-driven replication that is what we know in the open and the non-autonomous replication that is what has been hidden. The concept of non-autonomous replication by definition would be tumor replication that is not self-driven and is dependent on growth factors that only available from the host. Although remains to be proven in experimental system, our hypothesis based on available evidence and clinical observations has point that most likely connection between the two modes of tumor replication is through local inflammation created by chemotaxis from autonomous replicating tumor cells during active growth and replication [3]. Upon inhibition of autonomous replication by TKI drugs, support for local inflammation is terminated and the non-autonomous tumor replication is also terminated. This hypothesis can explain several unresolved mysteries associated with use of TKI drugs such as the mechanism and kinetics of drug resistance. The emergence of new mutations during the non-autonomous replication of majority of tumor cells would be the main source of this event and some tumors may contain more than one self-driven mutations to begin with and thus are “naturally resistant”. The significance of this theory/hypothesis on the mode of tumor replication is that it has looked grossly beyond the complicated and incomprehensible details of the genetic composition of each tumor and precisely into their actual proliferation and has made individual assessment for prognosis possible. In addition, the practical feature of this assessment is readily available to current

clinical practice in that even without a genetic testing, we can make accurate assessments. In this report, we describe our application of the assessment of tumor replication in two late-stage ovarian cancer cases to explain, retrospectively, past clinical history and to guide selection of future treatments.

The reason we have selected two ovarian cancer cases are several. First of all, late-stage ovarian cancer is notorious for bad prognosis for most cases, yet in some cases (25-30%) the cancer behaved not as progressively and the patients fared relatively good prognosis (>5 year survival after surgery) without knowing why. The accurate categorization of each case is difficult due to lack of molecular standards and most cases are staged according to TNM-based FIGO criteria [4]. These cases will provide one additional dimension to aid more accurate staging. Secondly, the similarities to these cases, as we have counted among our limited experiences with ovarian cancer cases, are surprisingly high, reaching 1/4-1/3 of all cases. Thus it is not a rarity but relates to a significant portion of ovarian cancer cases. The lessons from these cases should be relevant to the dealing of other similar cases in the future. Thirdly, due to the high death rates of ovarian cancer, a trend of applying more and more aggressive tumor reductive therapies has been observed. This, in our view and based on the cases described here, is counterproductive if used indiscriminately for all cases. We intend to use these cases to illustrate the need to apply individualized treatment for each case. Fourthly, as antitumor immunity is a significant factor affecting tumor replication and prognosis of all cancers including ovarian cancer cases, the overall ratio by which antitumor immunity plays significant role in ovarian cancer is low (<20%), leaving the role of tumor replication more influential and more distinctive. Through these cases, we will show that tumor can develop even without autonomous replication in ovarian cancer cases. The prognosis of such tumor is fare to good if not over treated. In cases of post-surgery metastasis there is always emergence of self-driven tumor cells. These cells may develop from post-surgery treatments, but despite they are self-replicating, the ability to metastasize seems limited and the clinical courses do not follow that of most aggressive recurrent ovarian cancer cases. The hidden mechanisms behind these cases will be further discussed.

Case Description

Case 1: A 42 year old women had developed large quantity of ascites in 2014. Hospital examination indicated large lumps (7-10cm) in the ovaries. The patient had history of ovarian cyst and was on hormonal supplement for several years. Tumor marker CA 125 was significantly elevated (>1000). Ovarian cancer was thus suspected. The case went for surgery and based on tumor invasion/distribution, a FIGO staging of 3C was assigned to the case. Accordingly, the patient went through adjuvant chemotherapy and accomplished 6 rounds of platinum-based therapy before entering into observational period. Tumor marker CA 125 dropped after surgery and chemotherapy to below 10 (normal range: 0-35). Nearly two years following surgery, tumor marker CA 125 began to rebound slowly and steadily but still remained in the normal range. The patient took action of 3 rounds of chemotherapy spaced 4 weeks apart till tumor marker returned below 10. The case remained unremarkable till she went to our advice in 2019 to seek for a retrospective view of her disease course and a future outlook. We examined her tumor sample from surgery for the mode of tumor replication as well as presence of any antitumor immunity. The observation indicated that this is a typical ovarian serous adenocarcinoma (Figure 1, HE). There was no definitive Ki-67 positive signal in the entire large tumor section (Figure 1, Ki-67). Instead, there was a very active proliferation signal by PCNA staining throughout the entire section (Figure 1, PCNA). No presence of antitumor activity was detected as there was a total lack of T cell presence in the tumor (Figure 1, CD3). These observations thus point to a situation of a non-autonomous replication-driven growth without involvement of antitumor immunity for post-surgery protection [5]. Based on this assessment, we attributed the relatively good post-surgery prognosis for this case to the lack of self-driven tumor replication. However, we also believed that the two incidents of elevated tumor marker during the observational period may reflect true recurrences at early stage. These recurrences were likely driven by autonomously replicating tumor cells not from the primary tumor, but residual tumor cells mutated by chemotherapy (see the next case). These recurrences, nevertheless, were caught early and eradicated by chemotherapy. By 2019, the case had survived disease-free post-surgery for more than 5 years, we believe that the case had reached clinical cure and there was no need for future intervention. The patient remains healthy today, more than 6 years after surgery.

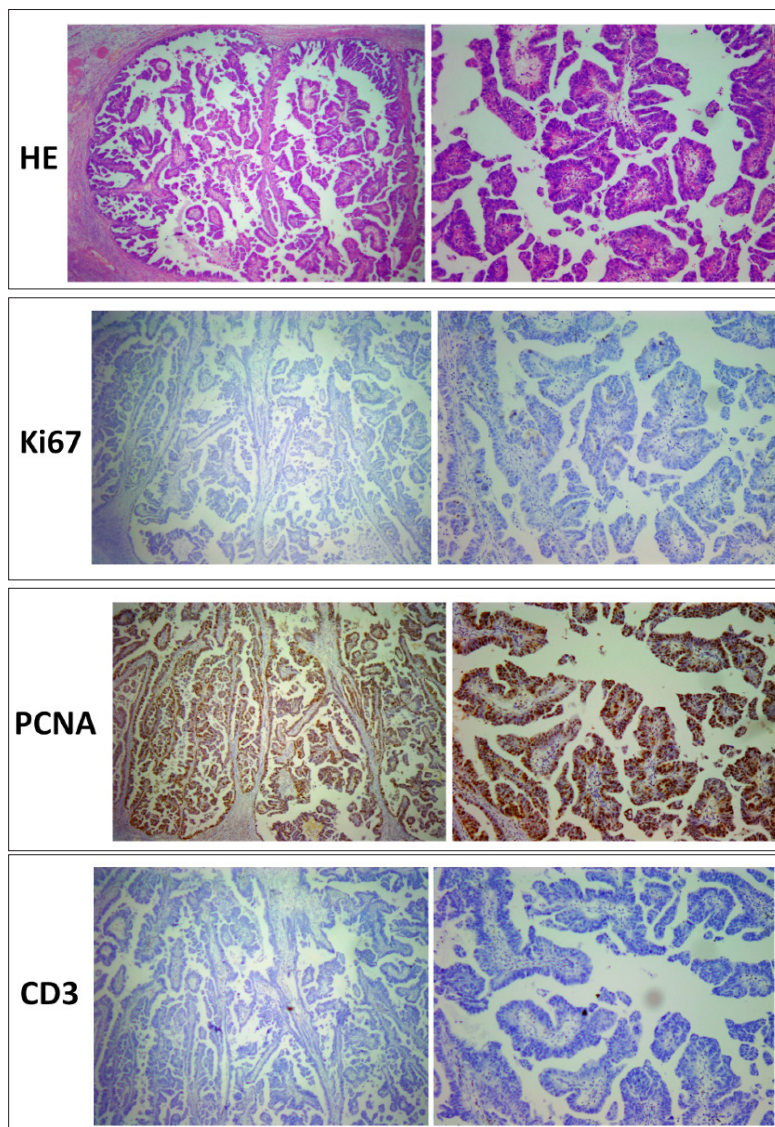


Figure 1: The mode of tumor replication and presence of antitumor immunity in Case 1. The sections from resected primary tumor were stained for HE, Ki-67, PCNA and CD3. Micrographs of low (40X) and high (100X) magnifications from representative area are presented.+

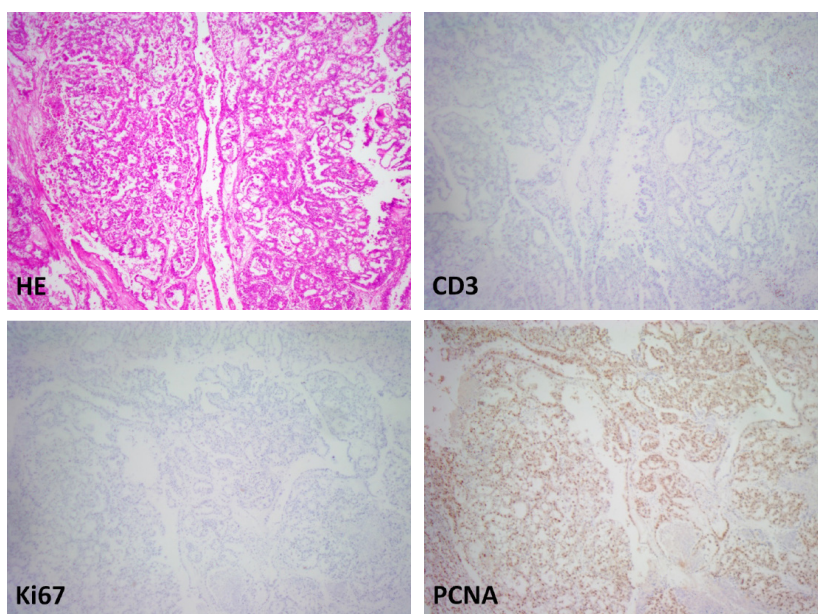
Case 2: A 46-year old woman went to hospital for persistent stomach discomfort in 2014. Imaging revealed a lump of 8.1x7.4x6.7 cm in the left ovary. Tumor markers were elevated (CA 125=125; CA199=80). The patient had long history (>10 years) of ovarian cysts. A case of ovarian cancer was suspected and the patient went for surgery. Pathological report showed a large (11cm) clear cell carcinoma of the left ovary and smaller tumor in the right. Tumor invaded surrounding organs but no tumor metastases were found in distant sites outside of pelvic cavity. A FIGO staging of 3C was assigned to the case. Post-surgery adjuvant chemotherapy was arranged. However, during chemotherapy, various small tumor nodules throughout peritoneal and pelvic cavities were detected by PET-CT. Another PET-CT examination 10 months after surgery (5 months after cessation of adjuvant therapy) showed active metabolic signal (SUV>8) in one small tumor nodule (1.3x0.8 cm) between liver and spleen among several other small and less active metastases throughout the peritoneal and pelvic cavities. The patient then resisted further chemotherapy and these previous metastases dissipated gradually without treatments. By 30 months after surgery, CT imaging showed the nodule between liver and spleen increased in size and was measuring 3.2x1.7 cm. This

recurrent tumor was resected by a second laparoscopic surgery with possible residual disease. Two more rounds of chemotherapy were arranged post-surgery. Six months following second surgery, with significant elevation of sensitive tumor markers, a recurrence of a nodule of 2.9x2.5 cm at the surgery site was confirmed by CT imaging. It was considered a recurrence due to incomplete surgery of the previous resection. A third and more extended surgery was again performed in January of 2019. Sensitive tumor markers (CA125 and CA199) returned to normal ranges after surgery and remained low thereafter. Imaging tests have since returned unremarkable findings. The patient then went to us to seek an assessment of risk for recurrence. We looked at all three samples from previous surgeries for clues to repeated post-surgery recurrence. Examination of the sample from the primary tumor showed that it was a clear cell carcinoma of the ovary (Figure 2A, HE). Tumor replication was not self-driven because there was no Ki-67 positive staining throughout the entire large tumor section (Figure 2A, Ki-67). Instead, active tumor proliferation was detected with PCNA staining (Figure 2A, PCNA), indicating this is a non-autonomous replicating growth despite the heavy invasive nature of the tumor. No presence of T cells was detected in the

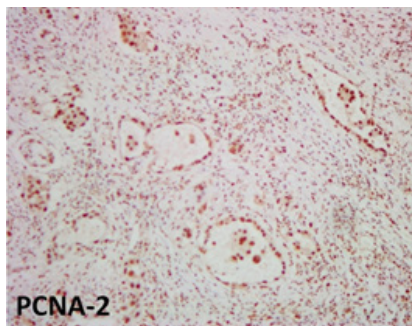
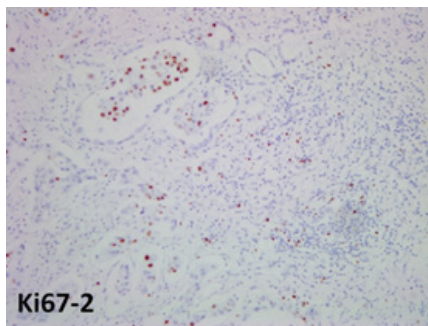
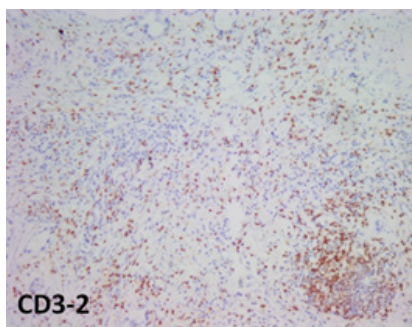
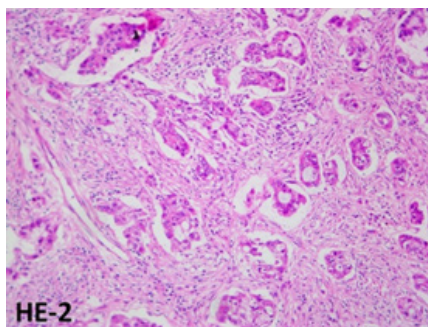
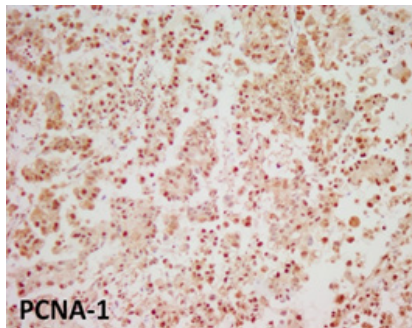
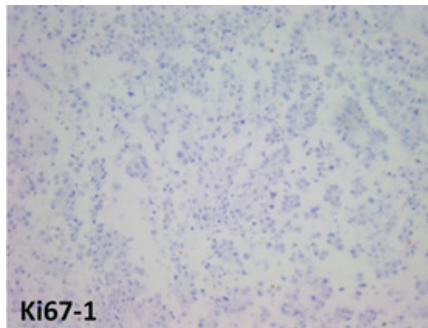
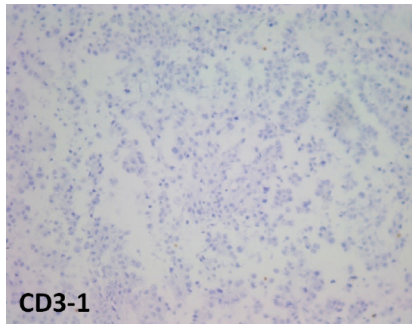
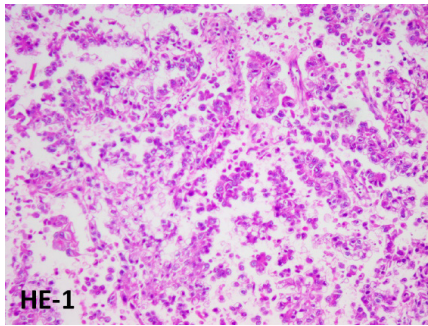
tumor, indicating there was no concomitant antitumor immunity in this case at that time. These observations would be consistent with the lack of metastasis in this case at the time of diagnosis and surgery, but inconsistent with post surgery recurrence at distant sites. We looked at the surgical sample from the second surgery for clue. The staining showed a metastasis with two distinct structures. Most of the resected tumor still showed a feature of a clear cell carcinoma, albeit structure-wise the metastasis drifted towards being more “compacted” in terms of intercellular space (Figure 2B, HE-1). Different from the primary tumor, the recurrent metastasis is self-driven for replication as ample positive staining for Ki-67 (averaging 30-50% labeling index) was seen (Figure 2B, Ki-67-1). Near 100% of tumor cells were also stained positive for PCNA (Figure 2B, PCNA-1). Also different from the primary tumor, T cells are present in the recurrent tumor, albeit not evenly distributed throughout the entire tumor. T cells are sporadic in tumor-active area (70% of the entire tumor, Figure 2B, CD3-1), but concentrated in group in the rest 30% area (Figure 2B, CD3-2). Associated with this presence, tumor structure was clearly altered (Figure 2B, HE-2) without loss of tumor replication (Figure 2B, Ki-67-2 and PCNA-2), reflecting immune attack and tumor destruction. These observations support the speculation that the metastasis was established by self-driven tumor replication not from the regular tumor cells in the primary tumor that replicated non-autonomously. The source of these autonomously replicating cells is not known, but is suspected to be associated with post-surgery chemotherapy (see Discussion section below). At the same time, with the appearance of

mutation(s) that drove autonomous replication, immune recognition took place and concomitant antitumor immunity developed. This immunity was not strong enough to control the progression of the entire metastasis, but co-existed with the metastasis and destructed part of the tumor continuously. With complete tumor removal, this immunity, although not strong in number, may form effective memory for protection against recurrence and metastasis [5]. But the second surgery was known incomplete and the subsequent tumor relapse indicated that this immunity was not able to eradicate the residual tumor. Based on our experience, previous antitumor immunity will likely resume and increase with tumor relapse. It was indeed so as the examination of sample from the third surgery showed (Figure 2C, CD3-2 compared with Figure 2B, CD3-2). The recurrent tumor drifted further into low differentiation with tumor cells packed tightly without clear structure (Figure 2C, HE-1). Tumor replication increased in activity, too. Higher labeling index of Ki-67 (50-70%) was seen (Figure 2C, Ki-67-1). Large number of T cells was found surrounding part of the tumor in a structure mimicking lymph node with germinal center (seen labeled with Ki-67 positive cells) and surrounding T cell zone (not shown). The T cell numbers and their activation state all showed enhancement compared to what was seen in the sample from the previous resection. With a complete surgery, this immunity was likely to form effective post-surgery protection against further recurrence and metastasis from this tumor. With these observations and interpretation of previous disease course, we reached the assessment that this case will likely enter clinical cure after the third surgery. The patient remained healthy today.

A



B



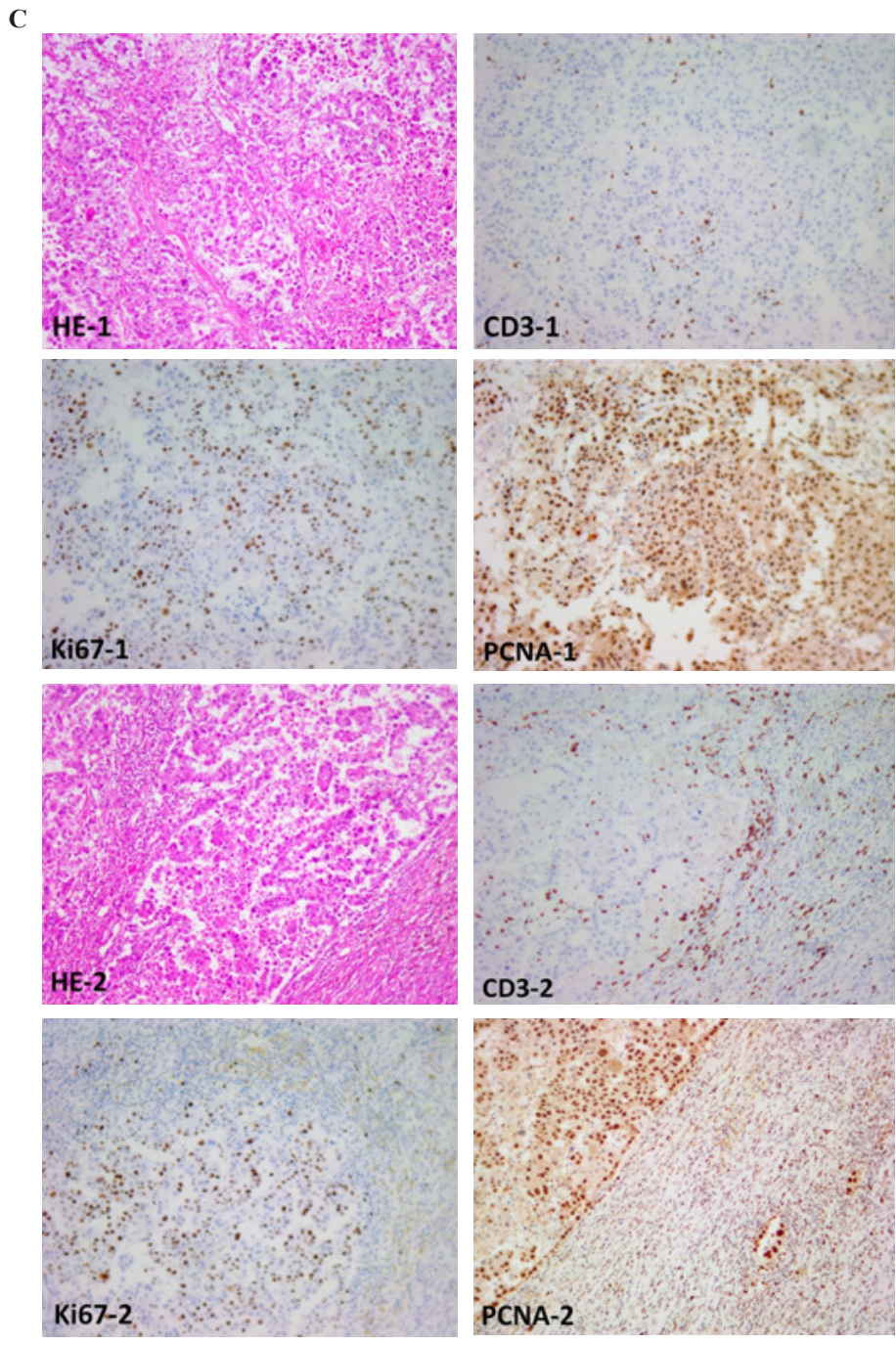


Figure 2: The mode of tumor replication and presence of antitumor immunity in the primary and metastatic tumors of Case 2. The sections from resected primary tumor (A) and first recurrent metastasis (B) and recurred metastasis (C) were stained for HE, Ki-67, PCNA and CD3. Micrographs of 100X magnification from representative area are presented

Discussion

Tumor replication is necessary for growth, yet not all tumors replicate in a steady and irrevocable manner as Dr. Dunphy had pointed 70 years ago [1]. Control of tumor replication is an essential part of antitumor therapy because regardless how much the therapy kills tumor cells, as long as there are remaining cells capable of replication, tumor always returns. That is the reason why cancer has not been successfully cured in general. But there are many cases of cancer that have been cured by clinical standards (recurrence-free survival). Surgery is the most reliable therapy for that because unlike any other tumor reductive therapies that rely

on individual cell killing, surgery extirpates the entire tumor with a clean margin without leaving residual cells at the site of surgery. But cancer surgery is still not curable in many cases even when clean resection is achieved. This is because distant metastases outside of surgery sites develop, and often more such metastases developed making future surgery impossible. By observation, cancer cases with known distant metastasis are most likely to recur after resection of primary tumor, thus is considered not suitable for surgery by any current clinical guidelines (for example NCCN). Many research works have focused on cancer metastasis with various discoveries and proposed measures to prevent metastasis

formation, yet there is no available therapy developed based on these discoveries. We have recently discovered that there are two modes of tumor replication called autonomous (or self-driven) and non-autonomous replication and the relationship between them [3]. Based on this categorization, it is deduced that only cells capable of self-driven replication shall be able to form distant metastasis. This hypothesis is supported by the cases presented in this report, especially by case 2 where distant metastasis had developed. In another metastatic ovarian cancer case (not presented here), we have seen a primary tumor without autonomous replication while the simultaneous supraclavicular metastasis showed ample self-driven replication. Our observations in all ovarian cancer cases where there was lack of autonomous tumor replication in the primary tumor from surgery, any metastases, either identified simultaneously with primary tumor or developed after surgery during recurrence, always contain ample (>20% Ki-67 labeling index) self-driven replication. Future observations will continue to test this correlation.

Ovarian cancer is the most lethal cancer for women. For most cases at diagnosis, it is already late stage with large tumor burden, severe local invasion and heavy ascites that inevitably facilitates tumor spread. Although about more than 70% late stage ovarian cancer cases recur after surgery regardless post-surgery adjuvant chemotherapy and die as a result, there are still about 25-30% late stage case survive more than 5 years after surgery. The reasons for the good prognosis in these cases have not been clearly identified. No previous reports have specifically looked into ovarian cancer cases without Ki-67 expression, although several reports have indicated a tight correlation between higher Ki-67 expression and worse prognosis [6-8]. Based on the prognosis of the two cases presented here, we argue that at least some of these ovarian cases with “good” prognosis may be due to lack of autonomous replication. Then how did these tumors grow without self-driven replication? In our recent report, we have proposed the connection between autonomous and non-autonomous replication in a tumor by local inflammation induced by production of autonomously replicating cells [3]. Inflammation is notorious for promoting tumor growth [9]. One common feature of these cases is local inflammation (ovarian cysts). We suspect that this condition had provided the necessary growth stimulation for the non-autonomously replicating tumor cells. Among the dozen late-stage ovarian cases we have seen in the past 6 years, there are 4 cases that showed this lack of autonomous replication in the primary tumor, a ratio between 1/4-1/3 of the cases. Whether this high ratio will hold in a large case pool remains to be seen, but even if only 10-20% of all late-stage ovarian cancer cases belong to this situation, we need to identify the case and treat them accordingly.

The emergence of distant metastasis and autonomously replicating tumor cells in the metastasis in Case 2 has raised the question about the source of this metastasis. Naturally, it could be from disseminated self-driven tumor cells from the primary tumor (like most other metastatic tumors). But we challenge this view because that being the case, we would expect multiple metastases to develop during the subsequent disease course of over 5 years. Looking back of the treatment history, we saw that multiple small metastases emerged first during post-surgery chemotherapy. These small temporary metastases may have either developed due to surgery-induced inflammation and wound-healing environment that are known to be tumor-promoting or they were stimulated by ineffective chemotherapy that may also be inflammation promoting. Most of these temporarily established tumor nodules, however, may not continue to grow due to subsequent killing

by chemotherapy and lack of growth factors after inflammation subsided. Subsequent course supports this interpretation. But chemotherapy drugs, as well-known carcinogens, may create new mutations that drive autonomous replication [10]. A similar situation from a previous case of lung cancer has been reported by us [3]. This is the reason why we suspected that this metastasis was induced by the mutagenesis effect of chemotherapy. As such, it is a rare event due to the nature of its creation. This indeed is supported by the subsequent disease course showing this was the only metastasis throughout time. As many late-stage ovarian cancer patients go through extensive adjuvant chemotherapy after surgery, the lesson from this case should be considered seriously.

The use of Ki-67 and PCNA staining to gauge autonomous and non-autonomous replication has not been established and accepted by the mainstream. Each marker has been studied before and their connection to prognosis has also been analyzed [6-8]. But to our knowledge, no previous study has compiled these two cell proliferation markers deliberately to measure different mode of tumor replication. PCNA is not a specific marker for non-autonomous tumor replication. Instead, it is a marker of all cell proliferation normal or cancerous, thus it will mark proliferating tumor cells regardless whether they are self-driven or by host factors. It is the Ki-67 marker that is likely to mark only proliferating cells with self-renewing ability (germinal cell). Its expression only indicates the growth is self-renewed, such as epithelial cells lining the digestive tract or germinal cells in the center of a lymph node. Whether the growth is cancerous is determined by whether renewable growth is at the same time regulated (for example by contact inhibition). This understanding will put tumor cells of both autonomous and non-autonomous replication into the category of cancer, but at the same time, will make the clear distinction that only self-renewable tumor cells can form distant metastasis. This new categorization on the mode of tumor replication thus provides a new angle to look at each cancer for more accurate assessment of the “growth propensity” for each case. It should be pointed out that the current practice of clinical description of Ki-67 labeling is grossly inaccurate in that not only a single % number cannot represent the actual situation of various regions of tumor cells in a tumor, the number is often overstated in many cases. Pathologists do not like to see lack of Ki-67 signal in a well-recognized tumor because they do not know what to make of that fact. Instead, they always believed that the staining process was faulty, and they try to “correct the wrong” by either over treating and over staining a sample, or they simply pick the “hot spot” in a sample to report as whole. This statement comes from comparing hundreds of pathology reports to our own staining. In our hands, the correct way to obtain true Ki-67 labeling is to use positive and negative controls to set the staining process constant. Once it is set, the lack of Ki-67 in some tumor sections should be taken as true feature of that tumor. When simultaneous staining of PCNA is carried out, the results are always interpretable when information from disease course is considered like we have done here with the two cases.

Conclusions

A substantial portion (25-30%) of late-stage ovarian cancer cases may be caused by non-autonomously replicating tumor cells. Regardless of their local invasiveness, these cases may have a good natural prognosis after tumor removal due to the termination of continued supply of growth factors for these tumors and the lack of natural ability to form distant metastasis. However, over intervention following surgery may change this natural course by promoting development of mutated tumor cells capable of

self-driven replication, thus form distant metastasis. Assessment of the mode of tumor replication should be carried out in each case of ovarian cancer to evaluate the risk of post-surgery recurrence. When autonomously replicating tumor cells represented by Ki-67 staining are present, the case is considered potentially metastatic. Otherwise, it may be entirely composed of tumor cells of non-autonomous replication. As such, they may not form distant metastasis naturally unless promoted by chemotherapy. In these cases, selection of post-surgery adjuvant therapy should be carefully planned.

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