Cell Proliferation and Tumors of the Central Nervous System Part 1:
Evaluation of Mitotic Activity

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Abstract. Evaluation of cell proliferation has been long recognized in pathology as a mainstay of diagnosis and important in the prognostication of a variety of neoplasms. Routine light microscopic evaluation of mitotic activity has long served as a reasonable assessment of cell proliferation. Counting mitotic figures has the advantage of being inexpensive and relatively quick. The main objections leveled against utilization of mitosis counts in diagnostic decision making are related to the instability of mitotic figures due to pre-fixation and fixation issues and problems with interobserver reproducibility of counts. This paper reviews factors that affect the identification of mitotic figures and the determination of mitosis counts. The role of mitosis evaluation plays in the evaluation of certain neoplasms of the central nervous system is discussed.

Key Words: Brain tumors; Cell proliferation; Mitosis; Mitosis counting.

The distinction of benignity from malignancy in neoplasia is dependent on a number of factors, most notably invasiveness, metastasis, and uncontrolled cell proliferation. Currently, there are number of methodologies available for the evaluation of cell proliferation in neoplasia. The most time-honored, traditional, and quickest approach involves an assessment of mitotic activity. This approach measures the number of cells present in the M-phase of the cell cycle (Fig. 1). In contrast, a number of alternate methodologies, including flow or image cytometric analysis, radiographic methods, and immunohistochemical analysis exist that allow for an assessment of the other proliferative phases of the cell cycle (G1, S, and G2-phases), which often account for the majority of proliferating cells in a given neoplasm. The relative merit of these other approaches versus mitosis counting, however, is dependent on the information one is seeking. Cognizance of exactly what one is evaluating and the limitations of each methodology are important to consider. One needs to weigh the cost and time that is required to perform these analyses and the additional information gained with the relative ease and low cost of simple mitosis counting. In everyday practice, the evaluation of a tumor for mitotic activity by routine histology remains the mainstay of cell proliferation analysis.

Proliferating cells, in a tumor or in normal growth, pass through an orderly sequence of events as outlined in the cell cycle (1) (Fig. 1). A variety of factors have been described in recent years that exert control at various points during this process, affecting a cell’s progress through the cycle or inhibiting a cell from entering into the proliferative phases of the cell cycle. For example, mean mitosis counts are generally higher among uterine smooth muscle tumors examined during the secretory phase versus the proliferative phase, suggesting a positive effect of progesterone on cell proliferation. Neoplasia is marked by a disruption of the cell cycle by any one of a variety or combination of mechanisms that may be stimulatory or inhibitory in nature, resulting in uncontrolled cell proliferation.

Quiescent cells or terminally differentiated cells reside in the G0-phase of the cell cycle. Cells that are not quiescent have the potential to enter the cell cycle, if properly stimulated (e.g. by growth factors). These cells first enter into the Gap1 (G1) phase, which is marked by cell growth and the production of components required for DNA synthesis. The next phase of the cycle is the S-phase, during which time DNA synthesis occurs and chromosomal DNA is replicated. This is followed by the Gap2 (G2) phase, during which time the cell ensures that DNA replication is complete and DNA damage is repaired prior to entering the M phase or mitosis phase of the cycle (2, 3). The entire cycle takes approximately 24 hours (h) to complete in rapidly dividing human cells (1). Beside loss of control of cell proliferation, tumors are characterized by abnormal chromosome numbers, possibly related to abnormalities in the “quality control” of the G2-phase of the cycle. Abnormalities that occur during the M phase of the cycle may result in abnormal separation and migration of sister chromatids to the opposite poles of the dividing cell.

During the mitosis (M phase) of the cell cycle, the dividing cell undergoes a series of events outlined in Figure 2. During the prophase portion of mitosis, the chromosomes become visible as extended double structures and the chromosome pairs become shorter and thicker. In metaphase, the chromosomes become aligned on the equator of the cell. During anaphase, chromosome pairs split and move toward opposite poles of the cell (Fig. 3a). In telophase the chromosomes reach their respective poles and soon after, the cell divides into 2 daughter cells, each with a complete set of chromosomes.
Cell cycle with proliferative phases marked by $G_1$, $S$, $G_2$, and $M$. $G_0$ corresponds to the quiescent phase.

Fig. 1.

Fig. 2. Schematic outline of the mitosis phase (M phase) of the cell cycle.

Mitosis counts have long been the gold standard by which we have routinely evaluated cell proliferation in neoplasms. Mitosis counts can be performed with relative ease, requiring only routinely processed, hematoxylin and eosin (H&E)-stained histologic sections and the ability to identify mitotic figures. The method is relatively inexpensive and yields fairly timely results.

The precise significance of identifying atypical mitotic figures is not well defined. The identification of abnormal mitotic figures is generally acknowledged to be a feature of malignancy, and in certain tumors, their identification may provide prognostic information (Fig. 3b) (4). The latter has not fortunately proved the case with CNS neoplasms, since the identification of “normal” versus “abnormal” mitotic figures histologically is prone to considerable interobserver variability and lack of reproducibility (4).

A number of factors may affect the mitosis counts generated. Several issues related to the fixation of tissue may theoretically be important in assessing number of mitotic figures observed in a given lesion. Delayed time to fixation, the temperature at which the specimen is stored prior to fixation, and the quality or type of the fixative may all potentially influence mitosis counts (5–11). Fixation delay of the tissue may be due to either retention of the specimen in the operating room for a long time or failure to promptly process the specimen in the surgical pathology suite. Other forms of fixation delay that typically do not affect neuropathology specimens, due to their relatively small size, are delays related to large specimens that are not sectioned prior to fixation, i.e., delays related to slow penetration of the fixative. Similarly, a delay in fixation can result from placing a relatively large specimen in a small amount of fixative.

The literature is divided with regard to the effect of delayed fixation on evaluating mitotic rates. Donhuijsen et al noted decreased mitosis counts after delays of fixation of 3 to 12 h (8). Mitosis counts performed by 2 observers after 3 h were 10% and 13% lower, respectively, than the initial value with immediate fixation. After 12 h, mitosis values were decreased in the 39% to 40% range as compared with the initial values. An increase in the number of pyknotic mitotic figures observed with delays in fixation accounted for decreased ability to identify the mitotic figures, a substantial factor in the apparent decreased counts (Fig. 3c). It did appear, however, that there was completion of the cell cycle in some cells as well as the identification of well-preserved mitotic figures after 12 h of delayed fixation, indicating that proliferative activity can continue after tissue is removed from the body. Cell cycle fractions by flow cytometry were also evaluated in tissue specimens that were delayed in fixation. Interestingly, there was not a substantial change between immediately fixed tissue and tissue in which fixation was delayed; only a slight increase in the $G_2 + M$ fraction was observed. Donhuijsen et al concluded that the “decrease” in mitosis counts associated with fixation delay was primarily attributable to inability to identify mitotic figures and was not, for the most part, the result of completion of cell cycle progression. Bergers et al
demonstrated similar findings evaluating breast cancer cases with delays in fixation up to 24 h (6). They similarly noted a poor quality of material with prolonged delays, resulting in an increased difficulty in being able to identify mitotic figures. There appeared to be some benefit to keeping the unfixed tissue at 4°C (refrigerated) as opposed to room temperature in reducing the accumulation of nuclear debris that obscured identification of mitotic figures.

Other studies have emphatically suggested that there is a true decrease in the number of mitotic figures one observes with delayed fixation; although, it is unclear in most of these studies whether harder to identify mitotic figures were included or disregarded in counting (7, 11–14). Graem and Helweg-Larsen observed a decline in mitotic index after delays in fixative of only 1 to 3 h in osteogenic sarcoma grown in thymic nude mice (11). They noted a relative accumulation of cells in the later phases of the mitotic cycle (more cells in anaphase and telophase versus prophase and metaphase) with delayed fixation, concluding that during this period the majority of mitosis moved toward completion.

Similar to delays in fixation, the type of fixative used may also make the recognition of mitotic figures more difficult. This is particularly true of fixatives that have a low pH such as mercury containing compounds and Bouin’s fixative. Such fixatives tend to cause increased tissue shrinkage and smaller sized nuclei (15). Inadequately buffered formalin may also have a lower pH, causing similar morphologic changes.

The quality of staining can also affect one’s ability to assess mitotic activity (16). Tissue section staining with H&E may cause difficulty in distinguishing pyknotic, folded, or hyperchromatic nuclei from mitotic figures. This problem has prompted some to suggest the use of alternative stains such as mucicarmine or Giemsa when obtaining an accurate mitotic count is important (17). Thickness of tissue sections also can influence the number of mitotic figures observed in a given microscopic field. With thicker sections, more cells are likely to be represented and the number of mitotic figures observed increased (5, 14, 18, 19). Thicker sectioning will also account for increased depth of field, requiring more depth of focus, making mitosis counting more difficult and a multidimensional exercise. Counts should ideally be performed at one depth of field range.

Methodologic differences in determination of mitotic counts also can result in significant variability. This is aside from the challenges associated with the “simple” recognition of a mitotic figure. How one assesses or counts mitotic activity can vary. Most of the commonly used approaches to evaluating mitotic activity express mitotic figures per a certain number of “high power fields.” Of particular note is the fact that the high power fields on various microscopes can vary considerably (Fig. 3d–f). In the examination of 26 different types of microscopes from 5 different companies, Ellis et al noted high power field areas to vary from 0.071 mm² to 0.414 mm² (20). Only some of these differences are explainable by differences in the magnification associated with the eyepiece. Therefore, recommendations for the use of certain threshold mitotic counts as criterion for diagnosis of a particular tumor type or grade ideally should include some definition of what is meant by a high power field i.e. the area of the high power field. This can easily be accomplished by determining the radius of the high power field using a micrometer and calculating the area using the formula: area = (pi) x (radius)². Unfortunately, many published papers and proposed mitosis count guidelines do not provide this information or the information is overlooked or disregarded in applying guidelines to one’s own practice.

For convenience sake, mitotic figures are assessed per 2-dimensional high power field. The fact that other non-tumor cells are present in the same field (e.g. endotelial cells lining vessel walls) and the area occupied by these cells can be variable from field to field, a more precise approach would correct for cell density by defining counts per number of tumor cells evaluated (21, 22). Such an approach would admittedly require increased time and would certainly be more tedious. This is the methodology typically employed for manually determining labeling indices using cell proliferation markers such as PNCA or Ki-67 (MIB-1).

The actual method of counting figures also may yield differing results. By convention, one begins counting in the areas of the tumor that have the most mitotic activity. Some report the single highest count observed per 10 consecutive high power fields after evaluating some arbitrary number of sets of 10 high power fields. Others have advocated counting 40 or 50 consecutive high power fields, beginning with and including the area with the highest count per 10 high power fields and then determining an average number of counts per 10 high power fields. Once counting has started in a certain field, the additional contiguous fields should be randomly selected and an attempt should be made not to maximize the count by selecting those areas with higher numbers of figures. Differences between these 2 main methodologies may be quite significant. In a study of 15 mitotically active smooth muscle tumors of the uterus, single highest counts ranged from 5 to 15 mitotic figures per 10 high power fields with a mean of 8.7 and median of 8.0 (23). Using the latter method of averaging, the mitosis counts ranged from 4.2 to 10.2 with a mean of 6.5 mitotic figures per 10 high power fields and a median of 6.2. The averaging method, as expected, should yield similar or lower counts.

Although it is generally agreed that the area of the slide with the most mitotic activity should be the focus of the
count, determining this is subject to considerable observer variability. Likewise, gross selection of portions of a larger tumor to sample is also subjective. Theoretically, a few randomly taken histologic sections of a large tumor may not necessarily be representative of the most proliferative area of the neoplasm. This underscores the importance of appropriately sampling large tumors in order to be assured that the highest grade or most aggressive features of the lesion are represented. This is particularly salient in tumors that are notoriously heterogeneous, such as gliomas. If mitotic figures were homogeneously distributed throughout a given tumor, counts from various sections could be expected to fit a Poisson distribution (24). Although this may be an accurate assumption for some tumors (i.e. tumors characterized by a homogeneous distribution of mitotic figures throughout), clearly, for many gliomas it is not.

The experience of the observer is critically important in the accurate identification of mitotic figures (16, 17, 25). Beside artefacts related to fixation and staining, care needs to be taken not to misinterpret such things as apoplastic bodies, neutrophils, mast cells, and formalin pigment for mitotic figures (5, 18, 26–28) (Fig. 3g, h). As a general rule, if one is uncertain about a particular structure, it should not be included in the count.

Despite the problems with interobserver reproducibility and the host of variables that may affect evaluation of mitosis counts and the identification of mitotic figures, the assessment of cell proliferation by this methodology has been used in a variety of histologic studies and is a significant component of a number of grading systems.

Most of the current grading approaches for astrocytomas rely on some assessment of mitotic activity. Both the modified Ringertz and World Health Organization (WHO) systems use a subjective estimate of frequency of mitotic activity, as opposed to specific count guidelines (29–33). Higher-grade tumors tend to have more mitotic activity than lower-grade lesions. The assessment of mitotic activity and the relative weight afforded such an assessment in these 2 approaches is somewhat subjective and common sense needs to prevail. It is not unreasonable to encounter an occasional mitotic figure in a low-grade astrocytoma (WHO grade II); this should not be interpreted as being indicative of a high-grade neoplasm.

Likewise, in a small biopsy, the absence of identifiable mitotic figures should not necessarily preclude one from making a diagnosis of anaplastic astrocytoma, providing the appropriate degree of cellularity and atypia is present. The more recently proposed St. Anne-Mayo approach to grading astrocytomas was an attempt at providing a more objective approach to grading astrocytic neoplasms (34). Tumors were stratified based on the presence of 4 histologic parameters, one of which was the identification of mitotic figures. According to the rules, the identification of a single mitotic figure is sufficient enough to increase tumor grade in most circumstances. Subsequent work has demonstrated that St. Anne-Mayo grade 3 astrocytomas, marked by nuclear atypia and a single mitotic figure, are no different in terms of survival from grade 2 tumors marked by nuclear atypia and no observable mitotic figures (35).

One of the significant problems in evaluating mitotic activity in astrocytic tumors is the fact that the lesions, by nature, are heterogeneous and the degree of heterogeneity clearly correlates with increased grade of tumor (36, 37). The extent of heterogeneity extends beyond the morphologic appearance of the lesion to include a host of other aspects of the tumor, including cell proliferation, karyotype, oncogene expression, expression of growth factors and growth factor receptors, and responsiveness to radiation therapy and chemotherapy (38–46). Consequently, the extent one samples a tumor and the location of the lesion sampled may impact one’s ability to identify mitotic figures. Even with a low-grade tumor, the more neoplastic tissue one has to review, the greater the likelihood that one will encounter a mitotic figure. From a practical standpoint, recognition of a mitotic figure in a hypercellular lesion, where the differential diagnosis is glioma versus gliosis, is evidence in support of a tumor diagnosis. In high-grade gliomas, care needs to be taken not to misinterpret mitotic figures in areas of vascular (endothelial) proliferation as evidence of tumor cell proliferation (Fig. 3i).

The evaluation of other glial tumor types and the role mitotic activity plays in these lesions is also somewhat nebulous. With regard to grading oligodendrogliomas and ependymomas, it is generally recognized that the higher-grade tumors are likely to have increased mitotic activity.

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**Fig. 3.** a: Mitotic figures in a metastatic breast carcinoma. A metaphase figure with chromosomes aligned on the cell equator (arrow) and a telophase figure (arrow). b: Atypical mitotic figure (arrow) in a metastatic breast carcinoma. c: Reduced identifiability of mitotic figures (arrows) with a delay in formalin fixative of 18 h. d–f: Anaplastic oligodendrogloma, differences in “high power fields” that are potentially observable given the make of microscope used: area of high power field in (d) = 0.15 mm², area of high power field in (e) = 0.2 mm², area of high power field in (f) = 0.25 mm². g: Apoptotic bodies (arrows) with pyknotic and hyperchromatic nuclei and hypereosinophilic cytoplasm may be misinterpreted as mitotic figures. h: Black formalin pigment (arrow) may also be confused with a mitotic figure. i: Mitotic figure (arrow) in a focus of vascular (endothelial) proliferation in a glioblastoma multiforme. All figures are generated from H&E-stained sections and original magnification “high power ×400”.

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The relative significance or weight afforded mitotic activity versus other histologic parameters in differentiating between low- and high-grade oligodendrogliomas and ependymomas is unclear. In some studies examining oligodendrogliomas, mitotic activity was not found to be of independent prognostic significance (47, 48). In contrast, Burger et al noted that mitotic activity was a prognostically significant factor in evaluation of oligodendrogliomas (49). In a comparison of the Smith and Kernohan systems (48, 50, 51), no mitotic figures were noted in the grades I and II categories. Tumors classified as a grade I and II according to the Kernohan system were distinguished from grade III tumors by the presence of mitoses and pleomorphic cells in grade III neoplasms (50). Currently favored grading approaches in oligodendrogliomas tend to stratify them into low- and high-grade lesions, with high-grade tumors being marked by “high mitotic activity” (52). Giannini et al supported this 2-tier approach, noting that both mitotic index and microvascular changes appear to be the most significant histologic parameters in predicting behavior, with mitotic indices of 6 or more per 10 high power fields being associated with significantly worse survival (53).

Similar to oligodendrogliomas, a variety of attempts at grading ependymomas have also been published (54–57). Again, there are discrepancies regarding the relative importance of evaluating mitotic activity. Similar to oligodendrogliomas, Rawlins et al noted that tumors that have “large numbers of mitoses” and vascular proliferation are more likely to behave in a more aggressive fashion (58). However, the exact amount of mitotic activity observed in a given tumor does not necessarily correlate with outcome (59); other histologic factors such as extent of surgical resection, location, and age of patient are of equal or greater importance (60–63). An interesting dilemma is proposed by the low-grade ependymoma that contains occasional small, markedly hypercellular and proliferative foci. The significance of this finding remains uncertain; however, it is not unusual to find increased mitotic activity and prominent cell proliferation, as evidenced by MIB-1 or Ki-67 immunostaining in these areas. Similar to oligodendroglioma, the currently favored grading approach to ependymoma is a 2-tier system. The WHO designates anaplastic ependymoma (WHO Grade III) as a tumor that shows “increased cellularity and brisk mitotic activity, often associated with microvascular proliferation and pseudopalisading necrosis” (64). Exactly what is meant by “brisk” in terms of numbers of figures is not defined and is subsequently subjective. Unfortunately, there is a lack of published data with sufficiently large numbers of cases to clarify this issue.

The role of mitotic counts and assessment of mitotic activity in the evaluation of astrocytoma variant tumors such as pilocytic astrocytoma, pleomorphic xanthoastrocytoma, and subependymal giant cell astrocytoma is not well defined. In general, pilocytic tumors and subependymal giant cell astrocytomas demonstrate very little, if any, mitotic activity. Similarly, the routine pleomorphic xanthoastrocytoma is typically devoid of much mitotic activity. In tumors that have considerable mitotic activity, this should serve as a red flag and caution should be taken before rendering a diagnosis of low-grade pilocytic astrocytoma or pleomorphic xanthoastrocytoma. In extraordinarily rare examples of pilocytic astrocytoma that have undergone malignant degeneration, the tumors are marked by increased mitotic activity and cellularity, prominent vascular proliferative changes, and foci of palisaded necrosis (65, 66). Pleomorphic xanthoastrocytoma is recognized as a higher-grade tumor by the WHO (grade II) and well described examples of malignant degeneration or progression of the tumor to a higher-grade lesion have been documented; the higher-grade tumors tend to be marked by increased mitoses and areas of necrosis (67–69). In a large series of 71 pleomorphic xanthoastrocytomas, the extent of resection and mitotic indices were the only 2 independent predictors of recurrent free survival by multivariate analysis (70). Only mitotic index was an independent predictor of survival, with tumors marked by 5 or more mitotic figures per 10 high power fields having a poor prognosis.

The most recent rendition of the WHO grading schema for meningiomas underscores the importance of assessing mitotic activity and actually presents precise guidelines with regard to the evaluation of these tumors (71). Specifically, the atypical meningioma (WHO grade II) is marked by increased mitotic activity, defined as 4 or more mitoses per 10 high power fields (high power field defined as representing 0.16 mm²). Likewise, one of the defining features of anaplastic or malignant meningioma (WHO grade III) is the presence of a high mitotic index, defined as 20 or more mitoses per 10 high power fields (high power field defined as 0.16 mm²). These definitions are directly based on data previously generated from the Mayo Clinic’s experience with these tumors (72, 73). Of the myriad of histologic factors examined by Perry et al in 1997, including sheeting, macronucleolation, necrosis, hypercellularity, small cell change, pleomorphism, nuclear atypia, and atypical mitoses, mitosis counts of 4 or more per 10 high power fields along the brain invasion were the strongest and most significant predictors of outcome (72).

In contrast to glial tumors where there has been a reluctance to proclaim a certain mitoses count as definitive of a higher-grade lesion, there has been a history of such approaches with meningiomas. Maier et al in evaluating 1,799 primary and recurrent meningiomas, stratified tumors into classic, atypical, and anaplastic categories (74). Maier defined atypical tumors by increased cellularity and at least 5 mitotic figures per 10 high power fields (high power field defined as ×400 magnification).
Anaplastic tumors were defined as lesions marked by "high mitotic rate"; a more specific guideline was not provided. Jääskeläinen and colleagues developed a 4-tier grading system for meningiomas based on the presence of 6 histologic parameters: nuclear pleomorphism, mitosis, necrosis, brain infiltration, loss of architectural pattern, and increased cellularity (75–76). Each of the parameters are scored from 0 to 3 representing absent, mild, moderate, and marked, respectively. The score for each of the 6 parameters is totaled and translated into a tumor grade. Precise definitions, though, with regard to mild versus moderate and marked mitotic activity were not provided. Mahmood et al further refined the criteria to provide guidelines for mitotic counts and grade with 1 to 2 mitotic figures per 10 high power fields corresponding to grade 1; 3 to 4 mitotic figures per 10 high power fields to grade 2; and 5 or more mitotic figures per 10 high power fields to grade 3 (77). Mahmood recommended counting mitotic figures in 50 consecutive non-overlapping microscopic fields and determining an average number of mitotic figures per 10 high power fields. Miller determined that tumors with mitotic counts equal to or greater than 1.1 per 1.96 cm² were associated with an odds for recurrence that was 22.7 times greater than the odds for tumors with counts below that cutoff (78). Xu et al suggested that mitotic indices greater than 6 per 10 high power fields was significantly correlated with the shorter disease-free survival (79).

Although providing precise guidelines for the determination of tumor grade is useful, the approach is not perfect. Definitions based on a given area, albeit appropriate, may be difficult to duplicate in real practice. The area of a high power field can be varied with different microscopes and technical adjustments in the actual area assessed should be made. This may be a problem, especially in borderline cases where the observed mitotic activity is near the threshold. In practice, there are likely many pathologists who do not know what the area of high power field is on the microscope that they are using. Although the WHO makes a precise recommendation regarding counts (i.e. with meningiomas), no indication of how one is to count is made (single highest versus average method). It is well known that meningiomas can grow rapidly during pregnancy and are prone to develop in association with certain malignancies such as breast carcinomas. This raises questions regarding the possible effect of the hormonal milieu of the tumor on mitosis counts, somewhat akin to uterine myomas.

For many of the remaining tumors in the CNS, the mitotic activity observed in the neoplasm roughly corresponds to the grade of the lesion. In lesions such as medulloblastoma, primary CNS lymphoma, and metastasis, which are often characterized by high mitotic activity, there is no additional prognostic benefit to the routine determination and evaluation of mitotic count. In other scenarios, such as the distinction between choroid plexus papilloma versus carcinoma, carcinomas demonstrate an increased mitotic activity (as expected), usually in the background of a constellation of other worrisome histologic features including nuclear atypia, brain invasiveness, necrosis, disorganized architectural pattern, and high nuclear to cytoplasmic ratio.

The recognition of mitotic figures (typical or otherwise) and the evaluation of mitotic counts remain the mainstay of routine diagnostic practice. The relative importance of identifying mitotic figures varies, depending on tumor type and the particular approach for a given tumor that one has chosen to adopt. Clearly, as with most other aspects of pathology, experience is important in the identification of mitotic figures and cognizance of the variables that may potentially impact on mitotic counts is important. The establishment of precise thresholds with regard to mitosis counts, albeit ostensibly useful and reassuring, are best viewed as rough guidelines given the myriad of factors that can influence the practical application of such recommendations.

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