What is the Cell Cycle?
A small proportion of cells in the human body are actively dividing, and these cells go through a series of events that enable them to grow and divide. The initial phase, termed interphase, and which is not associated with any morphological changes, allows the cell to replicate its chromosomes and prepare for cell division. The cell then leaves interphase, undergoes mitosis, and completes its division. The cells may also leave the cycle and be in a state of G0, during which normal cellular functions are undertaken. The interphase is further divided into G1, S, and G2 phases with specific molecular triggers (checkpoints) controlling the transition of cell division from one step to the other.

Ki67 and the Cell Cycle
The name Ki67 (pronounced Kee-67) is derived from the city (Kiel, Germany) where the protein was originally identified using antibody Ki67 (well #67) targeting the nuclei of the Hodgkin lymphoma cell line L428. It identifies a protein that is broadly expressed in all phases of the cell cycle (G1, S, G2, and mitosis), but is absent in resting (quiescent) cells (G0). Thus, Ki67 is used as a broad measure of proliferation. Ki67 labeling index has been documented to be prognostic in many cancers and is routinely used to assess proliferation in breast cancer and neuro-endocrine tumors. A number of kits and reagents are clinically used for the analysis of expression of the Ki67 protein.

Cyclin Dependent Kinases
Cyclin Dependent Kinases (CDKs) 4 and 6 specifically regulate cellular transition from the G1 phase of the cell cycle to the S phase. In the classical cell cycle model, a variety of stimuli lead to an increase in the levels of D-type cyclins which then bind to CDK4 or CDK6, and the complex(es) enter the nucleus and interact with a number of proteins including Rb1 (retinoblastoma). The binding of RB to CDK4/6 derepresses the activity of E2F family of transcription factors permitting the expression of its (E2F’s) target genes and the progression of cell cycle to the S phase. CDKs are themselves regulated by INK family (p16INK4A, p15INK4B, p18INK4C, and p19INK4D) and CIP/KIP family, which includes p27KIP1, p21CIP1, and p57KIP2 proteins. The intracellular regulatory processes are likely to be more complex and are not yet fully understood. Given the potentially powerful role of CDKs in the cell cycle, a number of attempts have been made to target these for therapeutic purposes.
CDK4/6 Inhibitors and Breast Cancer

CDK4/6 inhibitors can efficiently block the proliferation of cancer cells by inducing G1 cell cycle arrest. Cyclin-dependent kinase 4/6 (CDK4/6) inhibitors (palbociclib, ribociclib, and abemaciclib) have revolutionized the treatment for patients with HR+/HER2-negative (HER2-) advanced/metastatic breast cancer and have doubled median progression-free survival (PFS) as compared to estrogen modulation therapy alone in clinical trials. Furthermore, recent results from the monarchE randomized phase 3 clinical trial in chemotherapy naïve breast cancer patients have shown improved overall survival (OS) in patients treated with abemaciclib, a CDK4/6 inhibitor. The trial randomized patients on the basis of Ki67 expression (>20) to receive abemaciclib or standard of care endocrine therapy. Patients with high Ki67 had a significant improvement in progression free survival (Hazard ratio 0.696) as compared to those in the control arm. This is the first CDK4/6 inhibitor that has been FDA approved for adjuvant treatment of HR+/HER2- breast cancer.

Predictors of CDK4/5 Benefit in Breast Cancer (Prognostic and Predicative Clinical Implications)

As CDK4/6 inhibitors have been found to be therapeutically efficient, numerous studies have tried to identify predictive markers associated with the response. These studies have analyzed the expression of the target proteins, (CDK4, CDK6), Rb1, and p21, as well as a number of markers associated with the cell cycle. Unfortunately, there has been a poor correlation of response with any of these markers. As stated above, the phase 3 monarchE trial used Ki67 as a stratification marker and documented a high degree of benefit in patients with Ki67 > 20. In light of this result, the FDA has granted the Agilent Ki67 PharmDx assay, performed on the Omnis platform, the status of a companion diagnostic (CDx).

Controversies Regarding the Use of Ki67 as Companion Diagnostic (CDx)

a) Ki67 expression and response to Abemaciclib: The monarchE trial was a randomized clinical trial that enrolled high-risk HR+/HER2- patients as defined by clinicopathological features (cohort 1) or as defined by high Ki67 (cohort 2) (>20) based on the centralized analysis of Ki67 using the Agilent Ki67 PharmDx assay performed on the Omnis platform. In the patients enrolled in cohort 1, high Ki67 was prognostic, being associated with bad outcomes, however, it was not predictive of abemaciclib benefit. However, all patients in the trial (both cohorts) derived therapeutic benefit (patients with low Ki67 obtained benefit from abemaciclib (HR 0.704)). This has raised the issue as whether stratification may prevent some patients from getting therapy.

b) Pre-analytical variables: The Ki67 IHC is immensely altered by preanalytical variables such as type of biopsy, cold ischemia time, type of fixative, and fixation time. Additionally, Ki67 is also more sensitive to antigen decay with long-term storage in paraffin.

c) Reagents and kits: The FDA approval for the assessment of Ki67 requires the use of Agilent PharmDx assay on the Omnis platform. This is a new platform and is not widely available even in the USA. The performance of the assay on other Agilent platforms is not known, although there is preliminary data to suggest comparable results can be obtained using the link-48 platform. It is unclear at this time, whether the cutoff of 20% would be applicable to reagents and kits from other vendors, particularly as there is poor standardization of the antibodies and their concentrations used in the clinical labs.
d) Controls for Ki67: One of the main problems in the analysis of Ki67 is the lack of a negative control. Almost all tissues will have some proliferating cells that are highlighted by this antibody. This has resulted in the antibodies being used at variable dilutions resulting in different intensities of staining of the nuclei.

e) Consistency in Assessment of Ki67: Data from the International Ki67 in Breast Cancer Working Group (IKWG) has documented dramatic inter-observer and inter-laboratory heterogeneity in its assessment and has expressed serious concerns regarding its routine use. Over the last decade, these investigators have systematically analyzed the causes of variability and come up with guidelines regarding the assessment of this marker by immunohistochemistry (IHC).

**Best Practices and Recommendations for Assessing Ki67 in Early Breast Cancer**

There are several advantages of having a Companion diagnostic assay for the assessment of Ki67:

a) The kit requires the use of the assay under pre-specified conditions of fixation and processing, for example, the use of neutral buffered formalin and proscribed time for fixation.

b) The kit comes in a ready to use format; this will result in standardization of antibody concentrations, staining processes, and amplification systems.

c) The definition of what constitutes a positively stained nucleus is clearly stated. According to the FDA-approved criteria used in the clinical trial, a nucleus is considered positive for Ki67 if it meets the following criteria: 1) The signal must be unequivocally brown; 2) The staining must correspond to a nucleus, 3) The staining must cover the whole chromatin distribution within the nucleus; 4) The staining must correspond to viable, non-apoptotic cells. Of note, there is a distinction in this definition from that formulated by the IKWG which defines a nucleus positive if it is NOT blue; focal nuclear positive would be considered sufficient by the IKWG criteria but not by the clinical trial criteria. Grey nuclei are excluded in the FDA approved criteria.

d) The FDA-approved method requires the use of a “global” method for analysis. The whole slide is evaluated for the area of invasive tumor. Necrotic tumor areas, foci of carcinoma in situ, edge effects and fixation as well as processing related artifacts should not be scored.

e) At least 200 viable cells within the invasive component are assessed for expression of Ki67, and the results are expressed as a percentage of positive cells.

**Optimal Recommended Workflow and Communication between Pathology and Oncology**

Ki67 is routinely used for assessment of the behavior of breast cancer in many but not all institutions. The results of the monarchE trail have changed practice for our clinicians, and abemaciclib is now being increasingly used for the treatment of patients with HR+/HER2-breast cancer. With this change in clinical practice, and following discussions in multi-disciplinary tumor board, we have changed our standard reporting of biomarkers. At our institution, we have now started routinely assessing the expression of Ki67 and reporting it using the 20% cutoff. The testing is routinely performed on all core biopsies at the time of diagnosis as these are optimally preserved. We do not repeat the testing on the excision specimens. This transition has been easier for us as we have been using the Agilent
instrumentation and reagents in our IHC labs. For labs using other vendors and kits, a process of standardization and quality control/assurance will be required to ensure concordance with the 20% cutoff.

**Take Home Messages**

CDK4/6 inhibitors appear to fit the niche created by the documented lack of benefit of chemotherapy (see references to TAILORX and RxPONDER trials) for many patients with HR+/HER2- breast cancer. The expression levels of Ki67, using the Agilent PharmDx kit, appear to be the best biomarker for assessing the response to abemaciclib. It is critical for pathologists to strictly adhere to the scoring guidelines when evaluating companion diagnostic (CDx) assays. Additional work is necessary to identify correlates of PharmDx 20% cutoff with other Agilent platforms or with other reagents and detection systems. Lastly, while it is recognized that analysis of any marker by IHC is inherently subjective, and absolute concordance is nearly impossible to reach in spite of these limitations, one should strive to achieve precision while evaluating markers associated with selection of therapy.

**References**


