1 Rationale for Functional Profiling in Oncology

Cancer is the second leading cause of death worldwide. In 2020, the estimated numbers of new cases of invasive cancer and related death in the USA exceed 1.8 million and 606,000, respectively [1]. Since 1991, the cancer mortality rate has declined continuously in the USA, reaching an overall drop of 29% since 2017. The decline in cancer-related mortality is mainly attributed both to changes in smoking habits and to recent treatment advances. Nevertheless, the incidence of some cancers continues to rise, as is the case for cancers of the pancreas, liver and thyroid, while progress in treatment options is slowing for cancers amenable to early detection [1]. In addition, many cancer patients with advanced disease do not benefit from robust therapeutic options [2]; therefore, the development of effective anticancer treatment strategies is still an urgent medical need.

Over the last decade, molecular diagnostics has considerably promoted precision medicine in cancer through the identification of specific actionable mutations [3]. One of the first successes of precision medicine in solid tumor oncology was the discovery that a subset of non-small cell lung cancer (NSCLC) patients harboring specific somatic mutations in the epidermal growth factor receptor (EGFR) gene have a marked therapeutic response to EGFR tyrosine kinase inhibitors (EGFR TKIs) [4,5]. Although the majority of cancer patients have at least one molecular alteration, the percentage of advanced patients that have actionable alterations varies from approximately 10% to more than 50%, depending on the study cohort [2,6]. However, only a small subset of advanced cancer patients (13%) receiving molecular-targeted drugs experience an objective response [6]. Integrating tumor molecular markers, mainly genomic data, with functional profiling, namely, drug screening, will undoubtedly increase the therapeutic options and impact treatment outcomes. Ideally, personalized drug screening is performed with primary patient samples to provide rapidly efficient drugs with low toxicity.

In the first drug screening programs, initiated, among others, by Memorial Sloan Kettering in the 1940s, mouse models were used for screening potential anticancer agents [7]. In 1976, the Division of Cancer Treatment and Diagnosis (DCTD) of the National Cancer Institute (NCI) incorporated the use of human colon, breast and lung tumor xenograft models in the primary screening program of the Cancer Chemotherapy National Service Center (CCNSC). However, until the beginning of the 1980s, all of the molecules used in a drug screen were first evaluated in mouse leukemia models, leading to discovery of only a paucity of drugs as effective against solid cancers. This prompted the NCI to develop a screening program using human tumor cell lines [7].

It involved the application of two-dimensional (2D) culture conditions that helped identify some promising drug combinations, leading to new clinical trials [8]. However, only 5–7% of anticancer drugs developed from standard cell line screens in conventional 2D culture demonstrated success in clinical trials [9,10], and only 3.4% of phase I–III clinical trials investigating these anticancer compounds had degrees of success [11]. Considering the duration and cost of anticancer drug development, the low success rate of preclinical compounds in clinical trials calls for the use of more structurally relevant tumor models in drug screening to achieve a more reliable prediction of drug efficacy and toxicity.

The concept of patient-specific drug sensitivity was proposed decades ago. Hamburger and Salmon were among the first to propose an in vitro drug sensitivity test of anticancer agents for patient treatment [12]. Their work was based on studies carried out at the Ontario Cancer Institute in Toronto in the 1960s and early 1970s that showed the possibility of assessing the growth and chemosensitivity of some murine tumors in vitro using colony-forming assays in a semisolid medium [13,14]. Hamburger and Salmon developed this system at the University of Arizona Cancer Center to support the growth of human tumor cells [15,16]. In the mid-1970s, they proposed the human tumor colony-forming assay (HTCA) and tested it on samples from patients with multiple myeloma and various solid tumors [12,16,17]. This test has provided clear evidence of patient-to-patient heterogeneity in drug sensitivity and of its capacity to predict response to treatment [12,18,19]. In particular, in 1978, Salmon et al. reported their first results of correlation between in vitro chemosensitivity and in vivo tumor response of nine multiple myeloma patients and nine ovarian carcinoma patients. They observed a high correlation between in vitro drug resistance and a lack of clinical response, and some degree of concordance between in vitro and in vivo chemosensitivity [12]. In a retrospective study on 123 cancer patients, the HTCA displayed very good positive (0.88) and negative (0.94) predictive values when comparing the in vitro results of the test and the clinical responses [20]. These data are in line with other reports demonstrating that the HTCA could accurately predict drug resistance and, to a lesser extent, drug sensitivity [12,19,21–24]. Overall, the HTCA results suggest a 60–80% chance of clinical response if the patients are treated with a drug that inhibits colony formation in vitro. This percentage drops to 5–15% if the drug does not display an activity in the HTCA [25]. Von Hoff et al. compared the clinical outcome of patients with advanced malignancies after receiving a treatment selected empirically by the clinician or based on the results of a modified cloning assay. They observed that the partial response rate for the patients who received a test-directed individual therapy was significantly higher than the rate for patients who received the treatment selected empirically by the

clinician (21% versus 3%) [26]. However, while improving the response rate, the in vitro drug sensitivity assay did not improve patients' survival [26, 27]. Importantly, the feasibility of the HTCA was dependent on the ability of primary tumors to form colonies in soft agar. Unfortunately, not all tumors formed colonies in vitro, and the cloning efficiency (i.e. the number of colonies formed/number of cells plated) in this system was very low, ranging from 0.01% to 0.1% for many tumor types. These technical limitations combined with the scarcity of tumor cells in some clinical specimens resulted in the applicability of the HTCA for only 25% of cancer patients [19,20]. Nevertheless, for many years, the HTCA proved more predictive of an individual tumor's responsiveness to anticancer agents than other newly developed in vitro drug sensitivity tests, such as the radiometric system [28]. The HTCA was adopted by different research laboratories and laid the foundation for the development of improved drug sensitivity assays [24,26,29–32].

2 Tumor Models in Drug Screening

2.1 Two-Dimensional versus Three-Dimensional Cultures

The 2D cell culture is a simple, reproducible, convenient, rapid and cost-effective method for screening a large number of compounds. This system has significantly contributed to our understanding of cell behavior and drugs' mechanisms of action. However, it is becoming evident that 2D cell cultures cannot always accurately select clinically active molecules. They fail to mimic the three-dimensional (3D) tissue architecture, thus affecting, among others, the treatment response. Cancer cells are not the only determinants of a tumor's characteristics and behavior in vivo [33]. The tumor microenvironment (TME), namely the extracellular matrix (ECM), consisting of a network of proteins and proteoglycans, and the cellular components including, but not limited to, stromal fibroblasts, endothelial cells and immune cells, are key factors in cancer progression, metastasis and drug resistance [34,35]. Initial studies in ovarian cancer showed that cells cultured as aggregates are less sensitive to drugs than monolayer cell cultures [36,37]. Similarly, breast cancer cell lines cultured as multicellular spheroids were more resistant to paclitaxel and doxorubicin than the same cells grown in 2D conditions [38]. Further studies comparing the 2D and 3D cultures of colorectal cancer (CRC) cells revealed differences in signaling pathways and drug responses and showed that 3D cultures faithfully recapitulate the in vivo situation [39]. Many signaling pathways involved in chemoresponsiveness are differentially activated in monolayer cultures, which results in 2D cultures that are often (but not always) more sensitive to drug therapies, leading to false-positive screening data [37]. Evidence has been