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3D Anti-Cancer drug discovery models: A promising approach for precision medicine

104 – Biomedizin Innovativ – patientInnenfokussierte, anwendungs-orientierte sowie interdisziplinäre Forschung am Puls der Zeit

Abstract

Despite this paramount effort, attrition rates in cancer drug discovery are still alarmingly high. Research groups worldwide are recognizing that this phenomenon might be substantially associated with the lack of predictive pre-clinical drug discovery models. Complex 3D in vitro models might bridge the gap from non-physiological 2D models, still representing the gold standard, to costly, time- and labor-intense animal models. In the present study, we generated 2D and 3D non-small cell lung cancer (NSCLC) models and evaluated drug efficacies, oncogene addiction and cell survival in response to broad-spectrum cytotoxic agents and targeted inhibitors of the epidermal growth factor receptor (EGFR). The used cancer cell lines expressed either wild-type EGFR or harbored specific EGFR mutations that were shown to significantly influence EGFR inhibitor sensitivity in vivo. The major goal was to assess whether the EGFR mutation status would differentially influence drug efficacy in 2D and 3D models and whether the in vivo observed oncogene addiction of certain EGFR mutants can be recapitulated in any of these tested models. Our data clearly demonstrated that cancer cell physiology (e.g. invasive potential, gene expression, cell signaling) and drug responsiveness differed substantially between 2D and 3D cancer models. In the case of ErbB signaling we could show that only the 3D models (i.e. spheroids, tissue slices) exhibited a drug response equivalent to the clinic. In the conventional 2D monolayer culture the cancer cells exhibited only an attenuated drug response to the targeted therapeutics gefitinib, erlotinib and trametinib. Major changes in gene expression and altered ErbB phosphorylation in 3D cultures might significantly account for the differences in drug sensitivity. Taken together, the integration of complex 3D models such as spheroids and living tissue slices into preclinical drug discovery might reduce drug attrition rates in the near future.

Keywords:

Cancer Drug Discovery, 3D models, spheroids, tumor tissue slices, EGFR, Oncogene Addiction

According to the American Cancer Society's surveillance research report 2016 (*Lifetime Probability of Developing and Dying from Cancer for 23 Sites, 2010-2012*) the lifetime risk of developing cancer is 38% in females and 42% in males and statistically half of the cancer patients will die of it. Therefore, considerable effort is put in medical research for the discovery and development of new anti-cancer drugs. In the last decade cancer therapies are gradually integrating rationally designed drugs that specifically inhibit the activity of critical oncoproteins instead of broad-spectrum cytostatic and cytotoxic agents that cause excessive side effects (Alaoui-Jamali, Morand et al. 2015, Hutchinson, Johnson et al. 2015). Despite this paramount effort, attrition rates in cancer drug discovery are still alarmingly high (Kola and Landis 2004, Hutchinson and Kirk 2011). Research groups worldwide are recognizing that this phenomenon might be substantially associated with the lack of predictive pre-clinical drug discovery models. Nevertheless, monolayer-based cell culture models are still the gold standard in the early stages of preclinical drug development, even though the capability of these models to predict drug efficacy and safety is critically questioned today. Essential physiological parameters such as cell-to-cell and cell-to-matrix interactions, as well as 3D tissue architecture and the complex stromal microenvironment cannot be mimicked under these simplified culture conditions (Kunz-Schughart and Knuechel 2002, Clarke, Dick et al. 2006, Gupta, Chaffer et al. 2009, Gupta, Onder et al. 2009).

Complex 3D *in vitro* models might bridge the gap from non-physiological 2D models to costly, time- and labor-intense animal models (Jaganathan, Gage et al. 2014, Nath and Devi 2016). Being one of the best characterized and most promising cell-based 3D models spheroids feature high similarity to physiological tissues and demonstrated remarkable reproducibility in a broad range of cell-based assays (Smalley, Lioni et al. 2006, Hirschhaeuser, Menne et al. 2010). However, it remains controversial if 3D models are superior to conventional monolayer cultures in predicting oncogene addiction and drug efficacy (Amann, Gamerith et al. 2015, Ravi, Paramesh et al. 2015). More than a decade after the concept of oncogene addiction has been enunciated by Weinstein it is generally considered to be the Achilles' heel of cancer cells (Weinstein 2000, Weinstein and Joe 2008). This phenomenon that cancer cells strongly rely on the activity of a single oncogene for survival and tumor growth have led to the development of potent therapeutics targeting specifically these tumor drivers. ErbB proteins, particularly the epidermal growth factor receptor (EGFR), have been heavily investigated in the context of oncogene addiction due to their important role in the pathogenesis of many cancer entities.

In the present study, we generated 2D and 3D non-small cell lung cancer (NSCLC) models and evaluated drug efficacies, oncogene addiction and cell survival in response to broad-spectrum cytotoxic agents and targeted inhibitors of the epidermal growth factor receptor (EGFR). The used cancer cell lines expressed either wild-type EGFR or harbored specific EGFR mutations that were shown to significantly influence EGFR inhibitor sensitivity *in vivo*. The major goal was to assess whether the EGFR mutation status would differentially influence drug efficacy in 2D and 3D models and whether the *in vivo* observed oncogene addiction of certain EGFR mutants can be recapitulated in any of these tested models.

Since lung cancer accounts for more deaths than any other cancer entity we focused on non-small cell lung cancer (NSCLC), the most frequent lung cancer subtype accounting for approximately eighty percent. The used cell lines HCC827 (EGFR hypersensitivity mutation, oncogene addiction), NCI-H1975 (EGFR hypersensitivity mutation, resistance mutation), NCI-H1437 (EGFR wild-type, differentiated) and Calu-1 (EGFR wild-type, dedifferentiated/mesenchymal) were cultivated either as conventional monolayer, as floating spheroids or embedded in extracellular matrix (ECM), here referred to as organoids. First, by using immunofluorescent staining and phase-contrast microscopy, we assessed the epithelial differentiation status based on E-cadherin expression as well as invasion potential of the 2D and 3D cultures respectively. Thereby we could show that the invasive capabilities of the four NSCLC cell lines HCC827, NCI-H1975, NCI-H1437 and Calu-1 differed substantially. In addition, using FFPE samples of

spheroids we determined that the average cell size within spheroids is approximately three-times smaller than in monolayer culture. An observation frequently found in multicellular tumor spheroids *(Freyer and Schor 1989)*. After the treatment with the two EGFR inhibitors (EGFRI) erlotinib and gefitinib we assessed drug-related cytotoxicity in the four NSCLC cell lines using the alamarBlue viability assay. HCC827 cells which harbor EGFR mutations that render the cells hypersensitive to EGFRIs showed a dramatic difference in survival between 2D cultures (~50-60%) and 3D cultures (~5-10%). This remarkable reduction of cell viability in 3D may be ascribable to EGFR oncogene addiction. Therefore, by using the Caspase-Glo® 3/7 Assay we could determine that EGFRI treatment-related growth inhibition in HCC827 cells was indeed mediated by the induction of apoptosis.

NCI-H1975 cells exhibit two different point mutations T790M and L858R in exons 20 and 21 of the EGFR protein respectively. While the L858R mutation is known to hyperactivate EGFR signaling and therefore renders cancer cells EGFRI sensitive, the T790M mutation confers resistance against ATP analogs such as erlotinib and gefitinib (Bell, Gore et al. 2005, Shih, Gow et al. 2005). Interestingly, the inhibitors affected only NCI-H1975 spheroids while sparing 2D and organoid cultures. EGFR wild-type cell lines NCI-H1437 and Calu-1 did not respond to the EGFRIs, irrespective of their cultivation as 2D or 3D cultures. These results illustrate that drug sensitivity is different in 2D and 3D cultures.

On that basis, we tested three additional NSCLC cell lines HCC4006, HCC2935 and NCI-H1650 harboring EGFR exon 19 deletions. Strikingly, HCC4006 cells and HCC827 reacted similarly to both erlotinib and gefitinib and exhibited significantly reduced cell survival in 3D cultured cells (~30%). This indicated that oncogene addiction and cell signaling is substantially altered in 3D cultures (Cukierman, Pankov et al. 2002, Jacks and Weinberg 2002, Abbott 2003, Yamada and Cukierman 2007, Sharma, Haber et al. 2010).

We also determined the phosphorylation profiles of the EGFR in the different cell cultures. The phosphorylation levels of ErbB1 (Y825, Y992, Y1068, Y1086 and Y1148) and ErbB2 (Y1196) were in all cases significantly lower in 3D cultures. Only the expression level of ErbB3 was elevated in 3D cultures. At least 6 residues were described to serve as potential p85 binding sites which further lead to activation of PI3K/Akt signaling which strongly promotes cell survival and cell growth (Manning and Cantley 2007, Collins, Napoli et al. 2012, Roskoski 2014). Our preliminary data indicated that in 3D cultures ErbB3 is hyperphosphorylated at tyrosine 1197, a residue which is known to be involved in PI3K/Akt signaling.

To determine if differential drug sensitivities in 2D and 3D cultures were caused by altered expression of apoptosis-related genes we performed microarray analyses. Strikingly, a significant number of genes that are involved in the regulation of apoptosis were upregulated in HCC827 and NCI-H1975 spheroids. Using ingenuity pathway analyses we could identify critical signaling molecules involved in the regulation of apoptosis. In addition, we treated monolayer cultures and spheroids with gefitinib and performed RT-qPCR for 84 apoptosis-related genes. Particularly anti-apoptotic genes such as IL-10, BIRC5, BCL2A1 and BCL2 were significantly altered in 3D cultures after the treatment with the inhibitor gefitinib.

Our observations so far indicated that with increasing complexity of the *in vitro* model the predictability for drug sensitivity is increasing. The tumor microenvironment is a key factor critically influencing tumor progression and metastasis. Hence, we further developed 3D co-culture models consisting of NSCLC cells and cancer-associated fibroblasts that were embedded in natural extracellular matrices (ECM). After gefitinib treatment the cancer cells in the heterotypic co-culture model were found to be more sensitive to drug treatment than the cancer cells monoculture. Interestingly, cancer-associated fibroblasts significantly increased also the invasive potential of tumor spheroids of HCC827 and NCI-H1975 cells (Jacobi, Smolinska et al. 2016).

Apart from spheroid models we recently developed in a further approach living tissue-based drug discovery models (Jacobi, Smolinska et al. 2016). Thereby, we cultivated miniaturized tumor-tissue slices for a prolonged period of time *in vitro*. Immediately after surgery and tissue dissection, the slices retained the complex tissue architecture of tumors, and even showed signs of proliferation seven days after surgery. We additionally treated the tissue slices with kinase inhibitors and could confirm genotype-drug response relationships previously found in the clinical setting. Hence, this precision medicine model would allow to screen a compound library to find the most effective drugs against cancer cells exhibiting a particular genetic makeup.

Summarized, our data clearly demonstrated that cancer cell physiology (e.g. invasive potential, gene expression, cell signaling) and drug responsiveness differed substantially between 2D and 3D cancer models. In the case of ErbB signaling we could show that only the 3D models (i.e. spheroids, tissue slices) exhibited a drug response equivalent to the clinic. In the conventional 2D monolayer culture the cancer cells exhibited only an attenuated drug response to the targeted therapeutics gefitinib, erlotinib and trametinib. Major changes in gene expression and altered ErbB phosphorylation in 3D cultures might significantly account for the differences in drug sensitivity. Taken together, the integration of complex 3D models such as spheroids and living tissue slices into preclinical drug discovery might reduce drug attrition rates in the near future.

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